University of Alberta

Interactions of the Group B *Streptococcus* with Epithelial Cells: Host-Cell Signal Transduction Pathways in Group B Streptococcal Invasion and the Role of Surface-Associated Group B Streptococcal Phosphoglycerate Kinase in Pathogenesis

by

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"All we need to make us really happy is something to be enthusiastic about."

-Charles Kingsley

"Most people never run far enough on their first wind to find out they've got a second. Give your dreams all you've got and you'll be amazed at the energy that comes out of you."

-William James

Abstract

The Group B *Streptococcus* (GBS) is an opportunistic human pathogen that causes infection and invasive disease in newborns, pregnant women, and non-pregnant adults. GBS are able to invade a variety of non-phagocytic host cell types, including epithelial and endothelial cells. It is well established that the invasion of GBS into eukaryotic cells occurs in an actin-microfilament dependant process. The objective of this thesis work was to characterize host cell and/or bacterial factors involved in actin recruitment and manipulation by GBS. Initially, I focused on α-actinin, an actin binding protein closely associated with F-actin in the eukaryotic cell. This work showed that GBS does not recruit α-actinin, but that α-actinin antibodies bind to the surface of GBS, suggesting the presence of an actin-binding protein on the GBS surface. The potential actin-binding protein was subsequently identified as phosphoglycerate kinase (PGK). Treating epithelial cells with PGK prior to infection with GBS inhibited GBS invasion, suggesting PGK is important in the invasion process. It was also observed that PGK modified the actin cytoskeleton of epithelial cells. To characterize the effect of PGK on the host cell cytoskeleton and GBS invasion, the GBS PGK gene was cloned, expressed, and purified as a hexa-histidyl fusion protein (rGBS-PGK). This analysis suggested that surface-associated PGK contributes to actin manipulation and subsequent epithelial cell invasion by GBS. To study host-cell signal transduction processes involved in GBS invasion, the epithelial cell invasion process was mimicked using the HeLa 229 cell culture model in conjunction with chemical inhibitors to or dominant negative forms of a

number of host cell moieties. These studies identified that the Rho-family GTPases Rac1, RhoA, and Cdc42 are required for efficient invasion of HeLa cells by GBS. It was also found that GBS both requires and activates the phosphoinositide-3 kinase/Akt host cell signaling pathway during epithelial cell invasion. The work in this thesis addresses several facets of the GBS invasion process, including the host cell signal transduction cascade leading to epithelial cell invasion by GBS and a novel function for phosphoglycerate kinase, a protein on the GBS cell surface.

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List of Abbreviations and Units

α	Alpha
β	Beta
γ	Gamma
°C	Degrees Celsius
+ve	Positive control
6XHis	Six histidine residues
A	Absorbance
ABD	Actin binding domain
Abx	Antibiotics
ADP	Adenosine 5'-diphosphate
Akt	Protein Kinase B
Apaf-1	Apoptotic protease activating factor 1
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
BAP	Blood Agar Plate
BCIP	5-bromo-4-chloro-3 indolyl phosphate
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine Serum Albumin
C	Control
C-	Carboxy-
C3	C3 exoenzyme from *Clostridium botulinum*
CB	Carbenicillin
CDC	Center for Disease Control, Atlanta, Georgia
CFU	Colony forming units
CL	Cleared lysate
cps	Capsule biosynthesis operon
CPT	Camptothecin
DAS	Dense alignment score
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid

E	Eluate
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EF-Tu	Elongation Factor-Tu
EOD	Early onset disease
et al.	*et alli*; Latin 'and others'
F-actin	Filamentous (polymerized) actin
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
FT	Flow through
g	Acceleration due to gravity (9.8 m/s^2)
g	Gram(s)
G-actin	Globular (monomeric) actin
GAP	GTPase activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	Group A *Streptococcus* (Streptococci)
GBS	Group B *Streptococcus* (Streptococci)
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine triphosphate
h	Hour(s)
HBMEC	Human brain microvascular endothelial cells
HK-GBS	Heat killed Group B *Streptococcus*
HUVEC	Human umbilical vein endothelial cells
i.e.	*id est*; Latin 'that is'
ICIO	1D-6-hydroxymethl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan	Kanamycin
kb	Kilobase
KCl	Potassium Chloride
kDa	Kilodalton

L	Litre(s)
LB	Luria-Bertani
Lipo	Lipofectamine
LOD	Late onset disease
LPA	Lipophosphatidic acid
LTA	Lipoteichoic acid
M	Mole
m	Meter(s)
MB	Megabase
MEM	Minimal essential medium
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	Minute(s)
mRNA	Messenger RNA
MS	Mass spectroscopy
N-	Amino-
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NBT	Nitroblue tetrazolium
NFKB	Nuclear factor kappa B
Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical Density
P-	Phosphorylated
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK-1	Phosphoinositide dependant kinase 1
PDK-2	Phosphoinositide dependant kinase 2
PGK	Phosphoglycerate kinase
PH	Plextrin homology
PI3K	Phosphoinositide-3 kinase
PIP_2	Phosphatidylinositol 3,4-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein kinase B (also known as Akt)
PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
RER	Rough endoplasmic reticulum
rGBS-PGK	6XHis-tagged recombinant phosphoglycerate kinase from GBS NCS13 expressed in *Escherichia coli*

s	Second(s)
scPGK	Phosphoglycerate kinase from *Sacchromyces cerevisiae*
SD	Standard deviation
SDH	Surface dehydrogenase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBS	Tris-buffered saline
THB	Todd-Hewitt broth
ToxB	*Clostridium difficile* Toxin B
Tris	Tris(hydroxymethyl)aminomethane; 2-amino-2-hydroxymethyl-1,3-propanediol
U	Unit (measure of enzyme activity)
uPAR	Urokinase-type plasminogen activator receptor
WT	Wild-type

Mathematical Prefixes

M	Mega (10^6)
k	Kilo (10^3)
c	Centi (10^{-1})
m	Milli (10^{-3})
μ	Micro (10^{-6})
n	Nano (10^{-9})

Table of the Amino Acids

Amino Acid	Three Letter Code	Single Letter Code
Nonpolar (Hydrophobic)		
Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Phenylalanine	Phe	F
Tryptophan	Trp	W
Proline	Pro	P
Polar (Hydrophillic)		
Serine	Ser	S
Threonine	Thr	T
Cysteine	Cys	C
Tyrosine	Tyr	Y
Asparagine	Asn	N
Glutamine	Gln	Q
Charged (Negative) and Hydrophillic		
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Charged (Positive) and Hydrophillic		
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
Unknown or “other”	Unk	X

Chapter 1:

General Introduction

1.1. The Group B *Streptococcus*

The Streptococci are Gram-positive, catalase negative, non-motile, facultatively anaerobic organisms that grow in pairs or chains with each coccus having an average diameter of 1 to 2 μm (Fig. 1.1). This genus of bacteria causes a wide array of human infections, such as meningitis, pneumonia, pharyngitis, endocarditis, necrotizing facitis, and dental carries. However, many Streptococci are non-pathogenic, and some Streptococcal species are part of the normal flora of the human gastrointestinal tract, upper respiratory tract, skin, and mouth.

Rebecca Lancefield, a pioneer in the field of Streptococci, devised a scheme for streptococcal typing in 1933 when she observed that a soluble carbohydrate antigen could be recovered from the streptococcal cell wall following hot-acid extraction of the bacterial cells (Lancefield, 1933). These cell wall extracts were then assayed by immunopreciptiation with antisera from rabbits immunized with Streptococci isolated from a variety of sources such as humans, animals, and food. Dr. Lancefield's experiments lead to categorization of Streptococcal species into five subgroups, from A to E (Lancefield, 1933;Lancefield, 1934). Streptococci are also classified based on the hemolytic reaction they produce on solid blood agar medium (α, β, or γ hemolysis). In North America, sheep blood agar is commonly used as a solid culture medium for Streptococci and Todd-Hewitt Broth is commonly used for liquid culture.

The Group B *Streptococcus* (GBS, also referred to as *Streptococcus agalactiae*) forms a narrow zone of β-hemolysis on sheep blood agar. By definition, all GBS possess the group B cell wall associated carbohydrate antigen, as originally described by Lancefield in 1934 (Lancefield, 1934). Fig. 1.2 is a schematic of the GBS cell wall structure (Baron and Kasper, 2005). This antigen is not thought to be a virulence factor for GBS, nor is it important in natural immunity (Lancefield, 1975). It is, however, a useful tool to identify these organisms in the clinical laboratory. The Group B carbohydrate is made up of 4 sugars: rhamnose, *N*-acetylglucosamine, galactose, and glucitol (Michon *et al.*, 1987;Michon *et al.*, 1988;Michon *et al.*, 1991).

1.1.1. History

GBS was first isolated in the late 1880s from a case of bovine mastitis, and at that time GBS was not thought to cause infections in humans (Nocard and Mollereau, 1887). An association between GBS and human disease was not recognized until the 1930s, when reports of GBS causing sporadic cases of post-partum infections and death following childbirth appeared (Fry, 1938;Lancefield and Hare, 1935). At this time, GBS was regarded as causing only sporadic disease and did not garner much attention as a human pathogen. In the 1960s however, an increase in incidence of GBS neonatal disease appeared, and an awareness for the association between the presence of this organism and neonatal infection began to develop (Hood *et al.*, 1961;Mannik *et al.*, 1962;Reinarz and Sanford, 1965). By the 1970s, GBS was well recognized as a major cause of life-threatening neonatal disease (Baker *et al.*, 1973;Barton *et al.*, 1973;Franciosi *et al.*, 1973;McCracken, Jr., 1973), and since 1973, GBS has retained the notorious rank of being the number one cause of neonatal bacterial infections in the Western world (Fig. 1.3) (Schuchat *et al.*, 1997).

The cause(s) for the emergence of GBS as a neonatal pathogen and its persistent prevalence have never fully been understood. During the 1970s there was much speculation about factors contributing to the increase in GBS infections, such as antibiotic use, change in vaginal flora due to the increasing use of oral contraceptives, and use of intrauterine devices, but no definitive correlation between any of these factors and the sudden rise (and subsequent persistence) of GBS neonatal disease was ever reached (Baker and Kasper, 1977;McCracken, Jr., 1973).

1.1.2. Group B Streptococcal Disease

GBS can cause invasive disease in the newborn, pregnant women, and non-pregnant adults (Farley, 2001;Schuchat *et al.*, 1997;Schuchat, 1998;Tyrrell *et al.*, 2000), however, it is most widely known for its ability to cause neonatal infections. GBS neonatal disease occurring within the first week of life is termed Early Onset Disease (EOD) and usually presents as sepsis or pneumonia, and less often as meningitis or osteomyelitis. The major signs and symptoms that an infant has acquired GBS infection include lethargy, poor feeding, inability to maintain a stable body temperature or blood glucose level, and seizures (Dermer *et al.*, 2004). Late Onset Disease (LOD) is classified as GBS disease occurring in infants between one and twelve weeks of age. LOD is more likely to develop into meningitis than EOD, but LOD may also present as pneumonia, soft tissue infection, or urinary tract infections (Yagupsky *et al.*, 1991). Although meningitis is a common disease manifestation in LOD, infants who acquire LOD are less likely to be as severely ill as those with EOD (Yagupsky *et al.*, 1991).

It is thought the initial event to precipitate GBS infection in the newborn is for the neonate's exposed skin and/or mucus membranes to become colonized during delivery by GBS residing in the maternal urogenital tract, or by GBS that have ascended from the maternal genital tract into the amniotic fluid following rupture of the membranes (Anthony *et al.*, 1979;Schuchat and Balter, 2006). Most GBS colonized infants remain asymptomatic, but 1-2% go on to develop EOD, usually within the first few hours after birth (Schrag *et al.*, 2002;Schrag *et al.*, 2000). The presumed mode of infection is that the colonized infant aspirates the organism, allowing GBS access to the lungs. Once the pulmonary epithelial barrier has been breached and the organism is in the circulation, GBS will have systemic access and the ability to penetrate multiple anatomic sites (Gibson *et al.*, 1993;Nizet and Rubens, 2006;Rubens *et al.*, 1992). GBS may then gain access to the meninges, which is presumed to follow GBS invasion of brain microvascular endothelial cells (Nizet *et al.*, 1997;Shin and Kim, 2006). Direct fetal aspiration of GBS infected amniotic fluid may also contribute to development of pneumonia or sepsis (Schrag *et al.*, 2002).

The major risk factors for the development of EOD are maternal GBS carriage, a mother who has previously given birth to an infant with GBS disease, premature rupture of membranes, prolonged labor, and maternal GBS bacteruria during pregnancy (Schrag *et al.*, 2002;Schuchat and Balter, 2006). The risk factors for LOD are not as clearly understood, as this type of infection is presumed to be derived from nosocomial or community acquired sources (Schuchat and Balter, 2006). In the 1970s when GBS disease first began to appear in neonates, as many as 50% of cases were fatal, but since the 1990s the case-fatality rate dropped to 4%, presumably due to advances in neonatal care (Schrag *et al.*, 2000).

Approximately 10 to 30% of adult women are colonized with GBS (Schrag *et al.*, 2002). GBS have the ability to bind to human vaginal cells, and do so efficiently at an acidic pH characteristic of vaginal mucosa (Sobel *et al.*, 1982;Zawaneh *et al.*, 1979). Most of the time, GBS colonization is completely asymptomatic and no disease manifestations develop. In some cases, GBS does cause disease in pregnant and postpartum women, with manifestations including amnionitis, endometritis, sepsis, and rarely, meningitis (Aharoni *et al.*, 1990;Braun *et al.*, 1995;Pass *et al.*, 1982;Yancey *et al.*, 1994). GBS disease in this age group is rarely fatal.

In non-pregnant adults, GBS disease typically presents as soft tissue infection, bacteremia, pneumonia, sepsis with or without cellulitis, arthritis, endocarditis or urinary tract infection (Edwards and Baker, 2005;Tyrrell *et al.*, 2000) and most adult GBS infections are not related to pregnancy (Edwards and Baker, 2005). The most common risk factor for acquiring GBS infection in adulthood is being greater than 65 years of age; the incidence of GBS disease in elderly adults is 12 per 100 000 people in Canada (Tyrrell *et al.*, 2000) and 25 per 100 000 people in the United States (Edwards and Baker, 2005;Farley *et al.*, 1993;Farley, 2001;Schuchat, 1998). The American case-fatality rate for GBS infection in elderly adults is 15% (Edwards and Baker, 2005). Residing in a nursing home increases an individual's risk for acquiring GBS disease, as well as increasing the case fatality rate compared to community-acquired infection (Edwards and Baker, 2005). Another risk factor for acquiring GBS disease in adulthood is the presence of an underlying illness (such as diabetes mellitus) or being immunocompromised (Edwards and Baker, 2005;Farley *et al.*, 1993;Farley, 2001;Schuchat, 1998;Tyrrell *et al.*, 2000).

1.1.3. Prevention and Treatment

The Center for Disease Control (CDC) in Atlanta, Georgia has created a set of guidelines for physicians targeted at the prevention of neonatal GBS infection by delivering intrapartum chemoprophylaxis where appropriate. There are two broad approaches to identify candidates for situations where this may be warranted—the "risk based" and the "screening based" approach.

The risk based approach seeks to identify and deliver antibiotic prophylaxis to those women deemed to be at high risk for having an infant with GBS disease—the criteria include early delivery (prior to 37 weeks gestation), an intrapartum fever greater than 38 °C, or prolonged rupture of membranes (>18 h) (Schrag *et al.*, 2002). It is recommended that all women with GBS bacteriuria during pregnancy be given intrapartum antibiotics, as this is deemed to be highly indicative of a high GBS colonization burden (Schrag *et al.*, 2002). Antibiotic prophylaxis is also indicated in women who have previously given birth to a GBS infected infant (Schrag *et al.*, 2002). If no culture results are available at the time of delivery, the risk based approach should be followed (Schrag *et al.*, 2002).

The aim of the screening based approach is to identify and give antibiotic prophylaxis to GBS colonized women prior to delivery. Thus, it is recommended that all pregnant women be screened for GBS carriage at 35 to 37 weeks gestation and that women who are negative for GBS colonization at the time of screening not be administered chemoprophylaxis, even if the risk-based factors do present themselves during delivery. Correct collection and processing of screening specimens is necessary for the success of this approach, and collecting both vaginal and rectal specimens is recommended to enhance the probability of detecting GBS carriage if present (Schrag *et al.*, 2002). Further, the use of culture techniques designed to enhance the likelihood of detecting GBS if present, and minimize overgrowth of other flora (such as selective broth), are important for recovery of GBS in the screening process (Baker *et al.*, 1976;Dunne, Jr. and Holland-Staley, 1998;Schrag *et al.*, 2002).

Prior to the introduction of chemoprophylaxis regimens for GBS, the incidence of neonatal disease was approximately 2 to 3 cases per 1000 live births. Since the commencement of the active prevention campaign, this has dropped to 0.5 per 1000, with a corresponding decrease in the rate of postpartum GBS infections (Schrag *et al.*, 2002;Schrag *et al.*, 2000).

As GBS remain susceptible to penicillin, this is the recommended agent for intrapartum prophylaxis, with ampicillin being the next choice (Andrews *et al.*, 2000;Lin *et al.*, 2000;Schrag *et al.*, 2002). In the case of penicillin allergy, erythromycin and clindamycin are second line options for prophylaxis, however an increase in GBS macrolide resistance has been observed since the 1990s (Andrews *et al.*, 2000;Lin *et al.*, 2000;Schrag *et al.*, 2002). For treatment of GBS sepsis, penicillin or ampicillin combined with an amioglycoside is the treatment of choice.

1.1.4. Virulence Factors of GBS.

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The pathobiology of GBS infection indicates that this bacterium encounters a number of diverse host cell types, such as macrophages, epithelial cells, and endothelial cells during the invasive disease process. To overcome these defensive barriers and survive in the host, GBS must possess a variety of virulence factors. For most Gram-positive organisms, pathogenicity is a multifactorial process; this is also true for GBS. To some extent, GBS research to date has been largely focused on clinical and epidemiological issues, and research into GBS pathogenesis has lagged behind in comparison to many other organisms. However, in the last 10 years investigations into GBS pathogenesis has lead to characterization of some GBS virulence factors; these are described in this section (1.1.4) and are summarized in Table 1.1.

1.1.4.1. Capsule. The capsular polysaccharide serves as the basis for serological typing of GBS in the reference laboratory. As with other encapsulated organisms, the capsule is considered a major virulence factor of GBS. Nine capsular serotypes have been identified to date (Ia, Ib, II, III, IV, V, VI, VII and VIII). The majority of invasive neonatal disease is caused by GBS serotypes Ia, Ib, and III (Allardice *et al.*, 1982;Anthony *et al.*, 1981;Schuchat *et al.*, 1990). In North American adults, serotype V predominates, followed by III and Ia (Farley *et al.*, 1993;Schuchat *et al.*, 1994;Schuchat, 1998). Almost all GBS isolated from invasive cases are encapsulated.

A 15.5 kb region in GBS chromosome encodes the 16 genes for the capsule biosynthesis operon, *cps* (Chaffin *et al.*, 2000). The biochemical and immunological properties of GBS capsule have been characterized for almost all serotypes. Serotype III has been studied in the greatest detail, primarily because of its association with neonatal disease (Deng *et al.*, 2000;Jennings *et al.*, 1981;Rubens *et al.*, 1987;Wessels *et al.*, 1987). The capsular polysaccharide is composed of more than 100 repeating units of the monosaccharides galactose, glucose, *N*-acetylglucosamine and *N*-acetylneuraminic acid (salic acid) in a regular, repeating unit conformation. The repeating units range in length from five to seven residues, and the different serotypes are the result of different combinations of linkages and ratios of these sugars (Jennings *et al.*, 1983;Kogan *et al.*, 1996;von Hunolstein C. *et al.*, 1993;Wessels *et al.*, 1987;Wessels *et al.*, 1991) with the exception of serotype VI, which lacks *N*-acetylglucosamine (von Hunolstein C. *et al.*, 1993), and serotype VIII, where rhamnose is substituted for *N*-

acetylglucosamine (Kogan *et al.*, 1996). Experimental evidence suggests that the capsular polysaccharide is linked to the peptidoglycan backbone of the cell wall via the *N*-acetylglucosamine residues of the glycan (Deng *et al.*, 2000). Although structurally related, capsular seroptyes are antigenically variable.

The primary function of the GBS capsule is thought to be protection of the organism from phagocytosis by the host's immune system. In addition, the sialic acid component of the capsule inhibits activation of the alternative complement pathway by preventing deposition of active C3 on the GBS surface (Marques *et al.*, 1992).

To study the effect of encapsulation on pathogenesis, transposon mutagenesis has been used to create insertion mutations within the genes responsible for producing the serotype III capsule (Rubens *et al.*, 1987) and capsular sialic acid (Wessels *et al.*, 1989). These studies found that, in a neonatal rat sepsis model, an acapsular mutant exhibited a marked decrease in virulence in comparison to a wild-type strain (Rubens *et al.*, 1987) and that surface sialylation is critical for virulence, and aids GBS in evasion of host defense mechanisms (Wessels *et al.*, 1989). However, other investigations have shown that the presence of capsule decreases GBS invasion into a cultured lung epithelial cell line, A549 cells (Hulse *et al.*, 1993). This suggested that the capsule might cause steric hindrance, or that the presence of negatively charged sialic acid in capsule may cause repulsive forces between the bacterial and host cell surfaces, thereby inhibiting interactions between host cell receptors and adhesions on the bacterial cell (Hulse *et al.*, 1993). This suggests that capsule expression could be variable and phase switching may occur depending upon the organism's environment.

1.1.4.2. β-hemolysin. GBS characteristically exhibits a narrow zone of β-hemolysis on sheep blood agar; this is one of the first phenotypic features used to identify this organism in the clinical laboratory. The β-hemolysin is a pore-forming, non-immunogenic cytolysin that is active against a variety of cell types (Nizet, 2002) and it is produced by nearly 99% of all GBS isolates (Rubens *et al.*, 1990).

The β-hemolysin is firmly bound to the bacterial cell surface. It is very unstable in solution, and if extracted requires a high molecular weight carrier molecule, such as albumin, Tween-80, or starch to preserve extracellular hemolytic activity (Marchlewicz and Duncan, 1980). Consequently, β-hemolysin has not been purified to homogeneity. The 7 kb locus of the GBS chromosome responsible for hemolytic activity was first identified by Spellerberg and colleagues and was designated *cyl* in reference to its cytolytic activity (Spellerberg *et al.*, 1999a); this and subsequent studies revealed that the *cyl* operon is composed of twelve open reading frames (Pritzlaff *et al.*, 2001;Spellerberg *et al.*, 1999a). Pritzlaff and colleagues found that allelic replacement of the gene *cyl*E resulted in a loss of the hemolytic and cytolytic phenotype (Pritzlaff *et al.*, 2001). Additionally, *cyl*E was able to confer a hemolytic phenotype to a non-hemolytic *Escherichia coli* strain (Pritzlaff *et al.*, 2001). The GBS *cyl*E gene does not exhibit homology to other known bacterial toxins, suggesting that this moiety possess an novel and unique mechanism of action.

GBS β-hemolytic activity correlates with injury of lung epithelial cells (Doran *et al.*, 2002;Nizet *et al.*, 1996), erythrocyte membranes (Marchlewicz and Duncan, 1981), brain endothelial cells (Nizet *et al.*, 1997), and macrophages (Ring *et al.*, 2002b), as well as septic liver failure in New Zealand white rabbits (Ring *et al.*, 2002a). The injured cells exhibit membrane disruptions, cellular swelling, chromatin changes and lactate dehydrogenase release (Nizet, 2002). GBS β-hemolysin has also been shown to promote the induction of interleukin-8 (IL-8), a potent neutrophil chemotaxin (Doran *et al.*, 2002). In addition to being an initiating host signal of the innate immune response, IL-8 mediated neutrophil recruitment may also contribute to the destructive acute inflammatory processes seen in some cases of invasive GBS disease.

In 2000, Fettucciari and colleagues observed that GBS have the ability to induce apoptosis in macrophages and monocytes, but that when GBS are grown in conditions that inhibit synthesis or activity of β-hemolysin, apoptosis is also inhibited (Fettucciari *et al.*, 2000). These investigators proposed that β-hemolysin may promote apoptosis via pore-forming activity increasing membrane permeability, allowing for an influx of ions (including calcium) which appears to be a key factor in GBS induced apoptosis in macrophages. Thus, the β-hemolysin may also participate in evasion of the host cell immune system by GBS.

The damage resulting from the GBS β-hemolysin has several implications in terms of pathogenesis to the host. Direct injury of lung cells may contribute to development of pneumonia, which is characteristic in EOD. Epithelial damage could contribute to dissemination of the organism and systemic spread, helping to promote sepsis and meningitis.

1.1.4.3. CAMP factor. The CAMP factor is a 25 kDa protein secreted by GBS that works in concert with the sphingomyelinase from *Staphylococcus aureus* to produce the synergistic CAMP reaction, a unique "arrowhead" pattern of hemolysis observed on sheep blood agar when GBS are grown near colonies of *S. aureus*. This effect is mediated by *S. aureus* hydrolyzing sphingomyelin on the erythrocyte membrane to ceremide, leading to instability of the erythrocyte membrane and cell lysis upon exposure to the CAMP protein (Bernheimer *et al.*, 1979). The CAMP reaction has been used in the clinical laboratory as a diagnostic assay for GBS, and is named after the scientists who first described it in 1944, Christie, Atkins, and Munch-Peterson (Christie *et al.*, 1944).

While the CAMP reaction is a well established identification tool in the clinical laboratory, its role as a pathogenic determinant remains somewhat unclear. This is due in part to the fact that phenotypically CAMP-negative organisms have been isolated from cases of invasive disease (Hassan *et al.*, 2002), and the fact that the CAMP protein does not appear to cause red blood cell lysis on its own in the absence of staphylococcal sphingomyelinase (Bernheimer *et al.*, 1979). However, partially purified CAMP protein has been shown to exert lethal effects when administered to rabbits (Skalka and Smola, 1981), supporting the argument that CAMP does indeed function in GBS virulence. Additionally, the ability of the CAMP toxin to form pores in sheep red blood cells has been characterized recently (Lang and Palmer, 2003). Further studies to expand on this finding and relevance in other cell types are still required to elucidate the full physiological role of this toxin.

1.1.4.4. Lipoteichoic acid. Lipoteichoic acids (LTA) are polymers that are present in the cell wall of most Gram-positive bacteria. LTA extends through the peptidoglycan layer and attaches to the cytoplasmic membrane through hydrophobic interactions between the glycolipid anchor of the LTA and cytoplasmic membrane. Invasive GBS isolates have approximately five times more LTA on their surface than avirulent strains (Nealon and Mattingly, 1985). Strain-to-strain variation seems to be due to differences in LTA chain length, with GBS isolated from invasive cases possessing longer LTA chains than those isolated from cases of asymptomatic carriage (Nealon and Mattingly, 1985).

Various biological functions have been associated with this polymer, one of which being adherence of Gram-positive bacteria to eukaryotic cells. LTA has been demonstrated to adhere to cell membranes of erythrocytes and epithelial cells (Fischer, 1988). Pre-incubation of buccal epithelial cells with LTA prior to infection inhibits adherence of GBS to this cell type, but not to amnion cells, suggesting that host receptor specificity may be important for LTA mediated adherence (Nealon and Mattingly, 1985). Purified LTA from GBS is toxic to several cultured human cell types, such as HeLa cells, embryonic brain cells, and amnion cells (Goldschmidt, Jr. and Panos, 1984). A requirement for proper LTA anchoring has been demonstrated to be necessary for invasion of GBS into human brain microvascular endothelial cells (Doran *et al.*, 2005). Thus, although the full mechanism is not entirely clear, it appears that LTA has a role to play in GBS virulence.

1.1.4.5. Hyaluronate lyase. Hyaluronate lyase is a protein virulence factor of GBS encoded by the gene *hyl*B that can enzymatically degrade hyaluronic acid, a predominant component of the extracellular matrix of animal connective tissue and the nervous system (Pritchard *et al.*, 1994;Pritchard and Lin, 1993). This enzyme was initially thought to be a neuraminidase (Hayano and Tanaka, 1969), but this conclusion was later found to be incorrect when it was discovered that the enzyme cleaves hyaluronic acid at the 1,4-glycosidic linkage to yield *N*-acetylglucosamine and glucuronic acid (Pritchard and Lin, 1993). Although most hyaluronidases function by randomly cleaving hyaluron chains resulting in a mixture of oligosaccharide fragments, the GBS enzyme appears to travel processively along the chains of hyaluron, yielding primarily unsaturated disaccharide units (Lin *et al.*, 1994;Pritchard *et al.*, 1994).

Hyaluronate lyase has long been proposed to function as a spreading factor, destroying the normal connective tissue structure of the host (which is rich in hylauronic acid) and promoting bacterial dissemination. Both amniotic fluid and placenta, the main barriers of *in utero* infection, contain a high concentration of hyaluronic acid, so the enzyme may aid the organism in traversing this barrier. Milligan and colleagues have shown that a higher percentage of GBS isolates from invasive disease possess hyaluronate lyase compared to organisms isolated from asymptomatic carriage (Milligan *et al.*, 1978). However, a subset of hyaluronidase-negative GBS have been isolated from cases of infectious disease in both cattle and humans (Granlund *et al.*, 1998;Yildirim *et al.*, 2002). In a study by Granlund *et al.*, it was found that hyaluronidase expression was higher in GBS isolated from asymptomatic vaginal colonization than organisms isolated from cases of endocarditis. Therefore, hyaluronidase production may be more important for colonization and carriage than invasive disease (Granlund *et al.*, 1998).

1.1.4.6. C Protein. The C protein complex of GBS is well characterized and is composed of two antigens—the trypsin-resistant alpha antigen, and the trypsin-sensitive beta antigen. These surface-associated proteins have unrelated amino acid sequences and are produced independently of each other. Both antigens are anchored in the cell wall of GBS with the Gram-positive anchor motif LPXTG (Bolduc *et al.*, 2002).

The alpha antigen is frequently expressed in GBS, especially in serotypes Ia, Ib, II, and more rarely in serotype III; about 50% of clinical isolates assayed from these serotypes express the protein (Ferrieri and Flores, 1997;Nagano *et al.*, 2002). Antibodies to the alpha C protein have been shown to be protective for neonatal mice exposed to a lethal challenge dose of GBS (Michel *et al.*, 1991) and inactivation of the gene encoding the alpha C protein, *bca*, results in a five to seven fold decrease in lethality in the neonatal mouse model compared to the isogenic wild-type strain (Li *et al.*, 1997). The alpha antigen exhibits variation in the number of tandem repeats contained within the gene, and strains of GBS with alpha C proteins ranging from 62.5 to 167 kDa have been reported (Madoff *et al.*, 1996;Michel *et al.*, 1991). GBS strains isolated from maternal and neonatal pairs have been found to contain different numbers of tandem repeats, thus these deletions may be another mechanism of host immune evasion by GBS whereby reducing the number of repeats may reduce the ability of maternal alpha-specific antibody to opsonize a given GBS strain (Madoff *et al.*, 1996).

Purified alpha C protein binds to cultured cervical epithelial cells, suggesting a role for the alpha antigen in GBS adherence (Bolduc *et al.*, 2002). However, mutant strains with inactivation of the *bca* gene do not show any significant difference in ability to bind to epithelial cells compared to the wild type strain, although the wild type does invade with higher efficiency (Bolduc *et al.*, 2002). Thus the role of *bca* in pathogenesis is not completely clear (Bolduc *et al.*, 2002).

The main function elucidated for the beta antigen of C protein is the ability to bind to the Fc fragment of immunoglobulin A (IgA); the beta antigen possess two IgA binding domains, designated A and B (Jerlstrom *et al.*, 1996). The level of IgA bound by GBS is directly correlated with the level of beta C protein expression on the GBS cell surface, and strains isolated from invasive disease cases have the ability to bind IgA (Jerlstrom *et al.*, 1996). As IgA is the predominant class of antibody on mucosal surfaces, the ability to bind this immunoglobulin may be another mechanism for immune evasion by GBS.

1.1.4.7. Superoxide dismutase. A major strategy used by professional phagocytes to combat bacterial infection is the oxidative burst, whereby reactive oxygen intermediates such as superoxide anions, hydrogen peroxide, and hydroxyl radicals are produced. These reactive oxygen species can damage nucleic acids, lipids and proteins in the infecting bacteria. To protect against reactive oxygen intermediates, bacteria produce metalloenzymes such as superoxide dismutases (SODs) that convert superoxide anions to molecular oxygen and hydrogen peroxide. These may be metabolized by catalases or peroxidases, further protecting the organism from oxidative stress (Miller and Britigan, 1997). The manganese cofactored SOD of GBS, *sodA*, was cloned in 1997 (Gaillot *et al.*, 1997). In light of the fact that streptococci lack catalase activity, it was hypothesized that SOD may be required to protect GBS against reactive oxygen intermediates. A GBS *sodA* knockout mutant showed decreased ability to survive in the macrophage compared to the wild-type strain, suggesting that *sodA* is required for intracellular survival in this cell type (Poyart *et al.*, 2001). The *sodA* mutant also exhibited decreased virulence in the mouse model, was rapidly eliminated from the spleen and liver of the mouse, and had a dramatic reduction in the ability to survive in the blood and brain of mice (Poyart *et al.*, 2001). Based on this evidence, GBS SOD is considered to be essential for combating oxidative stress and plays a role in protecting the organism from host defenses (Poyart *et al.*, 2001).

1.1.4.8. C5a Peptidase. C5a peptidase is a multifunctional surface-associated protease that specifically cleaves and inactivates the neutrophil chemotaxin C5a, retarding host clearance of GBS by delaying or preventing recruitment of neutrophils to the site of infection (Beckmann *et al.*, 2002;Bohnsack *et al.*, 1991;Bohnsack *et al.*, 1997;Cheng *et al.*, 2002). Bohnsack and colleagues demonstrated that GBS C5a peptidase has the ability to inactivate C5a from human, bovine, and monkey sources, but not from pigs, rodents, or sheep (Bohnsack *et al.*, 1997). There is also evidence that C5a peptidase is an adhesion as it binds to fibronectin, part of the extracellular matrix of epithelial cells (Beckmann *et al.*, 2002;Cheng *et al.*, 2002).

C5a peptidase may have a role as a component in a GBS vaccine. Immunity resulting from antibodies to C5a peptidase does not have the limitation of being restricted by serotype specificity like capsular polysaccharide, because *scpB*, the C5a peptidase gene, appears to be antigenically conserved in all known serotypes (Cheng *et al.*, 2001). *In vivo* studies have shown that anti-scpB antibodies do not affect adherence of GBS to epithelial cells, but that internalization of the organisms into host cells is reduced (Cheng *et al.*, 2002). The C5a peptidase of GBS possesses very high sequence homology to that of other streptococci (such as Group A, C, and G Streptococci), suggesting that this protease plays an important role in the pathogenesis of all these organisms (Chmouryguina *et al.*, 1996).

1.1.4.9. Interaction with extracellular matrix components. The ability of Gram-positive bacteria to adhere to protein components of the extracellular matrix (ECM) of eukaryotic cells is regarded as an important factor in pathogenicity. The ECM serves as an underlying foundation of mammalian cells, and when tissue damage occurs, the ECM becomes exposed. Adhesion to ECM components contributes to colonization of mucosal and epithelial surfaces and can facilitate internalization of bacteria into host cells. The interaction of GBS with various ECM components is discussed in sections 1.1.4.9.1 through 1.1.4.9.4.

1.1.4.9.1. Fibrinogen. Fibrinogen is a 330 kDa glycoprotein that is found in high concentrations in plasma (Fuss *et al.*, 2001). The ability of GBS to bind to and interact with fibrinogen has been well characterized (Lammler *et al.*, 1983;Schubert *et al.*, 2002). Fibrinogen binding by GBS is mediated by the ligand FbsA, which is composed of repeating units 16 amino acids in length. Strain to strain variation in the number of repeating units exists (Schubert *et al.*, 2002;Schubert *et al.*, 2004). Inactivation of the *fbsA* gene results in loss of fibrinogen binding, and a marked decrease in the ability of GBS to grow in blood; this has been attributed to a loss of protection from opsonphagocytosis conferred by fibrinogen binding (Schubert *et al.*, 2002). Fibrinogen has also been shown to block activation of the alternative pathway of complement through inhibition of C3b binding.

1.1.4.9.2. Fibronectin. Fibronectin is a large, multifunctional glycoprotein which exists in a soluble form in blood and body fluids, and in a fibrillar form on cellular surfaces and within the connective tissue matrix where it participates in the maintenance of tissue integrity. Adherence to fibronectin is commonly observed in a variety of bacterial species. GBS strains isolated from human sources representing all serotypes were found to adhere to immobilized fibronectin (Tamura and Rubens, 1995). Other work has shown that C5a peptidase can act as a fibronectin adhesin (Beckmann *et al.*, 2002;Cheng *et al.*, 2002). In addition to C5a peptidase, *glnQ* (a glutamine transport gene that regulates the cellular glutamine level), is also required for fibronectin binding (Tamura *et al.*, 2002). It is unlikely that GlnQ participates directly as an adhesin, and it is proposed that modulation of glutamine levels in GBS results in reduced fibronectin binding ability (Tamura *et al.*, 2002). Further, although the mechanism is not understood, GBS strains possessing inactivated *glnQ* exhibit attenuated virulence in a neonatal rat model (Tamura *et al.*, 2002).

1.1.4.9.3. Laminin. Laminin is a major component of the basement membrane in various tissue systems and is important in the development and maintenance of cellular organization. Adhesion to the basement membrane is presumed to be an important event in bacterial colonization and invasion of damaged epithelial surfaces, and may facilitate translocation of a pathogen into the blood or cerebrospinal fluid. A GBS protein mediating attachment to laminin, Lmb, has been isolated, and mutants lacking the *lmb* gene show a marked reduction in laminin binding compared to the wild-type strain, suggesting the laminin binding protein functions as an adhesin in GBS-basement membrane interactions (Spellerberg *et al.*, 1999b).

1.1.4.9.4. Cytokeratin 8. GBS have been shown to bind to cytokeratin 8 immobilized onto polyvinylidene difluoride membranes (Tamura and Nittayajarn, 2000). However, cytokeratins are typically localized to the cytoplasm of host cells and thus the role of this interaction remains unclear. It is likely not involved in the process of GBS attachment to host cells, but may allow the organism to interact with the host cell cytoskeleton during the invasion process (Tamura and Nittayajarn, 2000).

1.1.4.10. Pili. Pili are proteinacious filaments that extend out from the microbial surface, and can facilitate numerous functions, including bacterial conjugation, transfer of bacterial effector molecules, adhesion, and host cell colonization (Sauer *et al.*, 2000). Although pili have been long recognized and well studied in Gram-negative bacteria (Sauer *et al.*, 2000), until recently the commonly accepted view was that Gram-positive organisms do not possess such structures. This dogma is, however, shifting.

In 2005, examination of GBS genome sequences by Lauer *et al.* revealed the presence of gene sequences with homology to pili, and electron microscopy studies revealed the presence of pilus-like structures extending from the GBS surface, protruding beyond the capsule (Lauer *et al.*, 2005). This was the first report in the literature of the presence of pili in a Gram-positive human pathogen.

GBS pili are made up of three proteins, each possessing an LPXTG motif for surface localization (Rosini *et al.*, 2006). Multi-strain genome analysis has revealed that every GBS strain sequenced to date possesses genes for pili (Buccato *et al.*, 2006;Rosini *et al.*, 2006). Allelic replacement mutagenesis of GBS pilin proteins suggests that GBS pili participate in attachment to and invasion of brain endothelial cells, and thus may contribute to the ability of GBS to cause meningitis (Maisey *et al.*, 2007). GBS pili can be expressed and assembled in *Lactococcus lactis*; preliminary evidence suggests this could be used as a delivery tool for a live GBS vaccine that triggers both systemic and mucosal immunity (Buccato *et al.*, 2006). The GBS pili present a very enticing topic for future study and it is likely that GBS pili will continue to be studied in detail, both to elucidate their role in pathogenesis and invasive disease, and as a vaccine target.

1.1.5. Genome Sequencing and Vaccine Development

Following implementation of the CDC guidelines for prevention and treatment of neonatal GBS infection in 1996, the mortality rate due to GBS in newborns dropped to 5% by 1999, where it has since plateaued (Dermer *et al.*, 2004). While this is a staggering improvement from the 1970s when the mortality rate following GBS infection was nearly 55%, neonatal GBS infection and consequently death still occur. Further, intrapartum screening and chemoprophylaxis for GBS only addresses the issue of EOD, and has not had any impact on the incidence of LOD, nor does it address the problem of GBS disease in non-pregnant adults. Thus, ongoing efforts have been targeted at the creation of a vaccine to prevent GBS infection. It is hoped that the development of such a vaccine would lead to eradication of human GBS disease in the long term. Immunoprophylaxis has the potential to address the various demographic groups affected by GBS, and presumably IgG directed against GBS in a pregnant woman would have the ability to cross the placenta and protect the baby as well.

Initial attempts at generating a GBS vaccine were focused on using capsular polysaccharide as the immunogen. However, purified GBS capsular polysaccharide has been found not to be highly immunogenic. Thus, the next strategy was to create vaccines based on full length capsular polysaccharide coupled to tetanus toxiod to improve antibody response (Kasper *et al.*, 1996;Paoletti *et al.*, 1990). A tetravalent preparation was created against serotypes Ia, Ib, II, and III which provided protection in the mouse maternal immunization-neonatal challenge model of GBS infection (Paoletti *et al.*, 1994). Unfortunately, coverage generated by such vaccines is limited; vaccine preparations made for a particular polysaccharide serotype do not confer cross-protectivity to other serotypes. An ideal GBS vaccine would not be restricted by serotype.

The focus now has shifted to a multivalent GBS vaccine which would combine multiple polysaccharide antigens and immunogenic GBS surface proteins. Several surface protein candidates have been proposed, such as Rib or C protein, but these moieties also exhibit some serotype specificity. For example, serotype III GBS rarely possess C protein, and this serotype represents a significant proportion of GBS isolates causing neonatal infection. An ideal protein candidate for inclusion in the GBS vaccine would be both highly immunogenic and conserved between serotypes. Thus, GBS genome sequencing projects were commenced with the goal of identifying possible proteins meeting these criteria. The concept was that, armed with genome information, an approach of "reverse vaccinology" could be performed whereby computer assisted identification of putative surface proteins would facilitate more timely identification of possible vaccine candidates, and also circumvent the technical problem imposed by traditional approaches whereby some genes are expressed differently *in vivo* than *in vitro*.

In 2002, 68 years after Lancefield published her work (Lancefield, 1934), the genomes of a serotype III (Glaser *et al.*, 2002) and a serotype V strain (Tettelin *et al.*, 2002) of GBS were sequenced in entirety (Fig. 1.4). Shortly thereafter, a serotype Ia genome sequence was available in an on-line database. Subsequent to these initial genome sequencing projects, eight more GBS strains were sequenced, with strains representing serotypes Ia, Ib, II, III, and V (Maione *et al.*, 2005).

Tettelin and colleagues analyzed the genome sequence information found in the 2.2 MB chromosome of GBS in an attempt to quantify the GBS "pan genome" (Tettelin *et al.*, 2005). They found that there is a GBS "core genome" made up of approximately 1800 genes (about 80% of the genomic information for each isolate) and a "variable genome" comprised of about 765 genes that are not present in all strains (Tettelin *et al.*, 2005). Further, in every new strain that was sequenced, unique genes continued to be discovered. The genetic diversity observed was not serotype dependant and serotype designation did not correlate with genetic similarity (Maione *et al.*, 2005;Tettelin *et al.*, 2005).

Multi-strain genome analysis by Maione *et al.* suggested the lack of a universal GBS vaccine antigen, and thus these investigators sought to identify vaccine candidate proteins that could be used in combination to give broad coverage. Computer programs were used to predict surface proteins and secreted proteins. This analysis also revealed that in addition to genetic differences, the surface accessibility of some GBS proteins, and thus the accessibility of antibody to epitopes, can differ between strains, even if the coding sequences are apparently the same (Maione *et al.*, 2005). Thus, while strides continue to be made towards GBS vaccine development, a definitive answer for this problem still does not exist.

If an effective and broad spectrum GBS vaccine is created, several issues regarding the vaccination protocol remain such as what would be the most effective context in which to administer the vaccine (*i.e.* would it be most effective to give to adolescent girls? Women of childbearing age? Older adults?). It is also unclear for how long following vaccination host immunity would persist.

The Group B *Streptococcus* is a major human pathogen and public health concern. While advances have been made regarding the biology and virulence of this organism, there are still many unanswered questions. Ongoing investigations into GBS may lead to new developments to combat and prevent infection due to this organism.

1.2. Bacterial invasion of eukaryotic cells

For a bacterial pathogen, adherence to host cells or host surfaces is usually the first step towards the invasive disease process. This initial adherence is usually followed by subsequent internalization into host cells. Internalization may occur via phagocytosis or bacteria-induced endocytosis. Non-phagocytic cells do not usually take up large particles, but several bacterial pathogens have evolved a mechanism to manipulate the host cell to facilitate this process (reviewed in Finlay and Cossart, 1997;Gruenheid and Finlay, 2003).

Great advances have been made in recent years regarding bacterial invasion processes, owing largely to the availability of new techniques and information such as genome sequencing projects, improved crystallography methods, and huge leaps in overall understanding of cell biology. The intention of this section of the introductory literature review is to provide a context as to why the field of bacterial invasion is of great value to study. More detailed descriptions of specific host cell signaling molecules and signal transduction pathways are provided in subsequent chapters relating their relevance to the GBS invasion process (Chapters 5, 6, and Appendix I).

The general paradigm for the process of bacterial invasion into host cells is that the bacterium interacts with certain receptors on the target host cell surface, which induces a series of signaling events in the host cell (such as modulation of host cell protein phosphorylation or phosphoinositide metabolism). This perturbs the host cell in such a way as to make conditions favorable to facilitate bacterial entry into the host cell. Manipulation of cytoskeletal remodeling is a common target of pathogenic bacteria to facilitate this process. In addition, bacterial interaction with the host cell may also up or down regulate certain host cell receptors to the pathogen's advantage. A variety of molecules, both on the bacterial side and the host side have been demonstrated to participate in this process, including proteins, carbohydrates and glycolipids. In addition to the ability to invade into non-phagocytic cells, some bacteria have evolved mechanisms to prevent uptake by professional phagocytes.

The mechanisms of host cell invasion have been characterized to a high level of detail for some bacterial pathogens, such as *Salmonella*, *Shigella*, *Yersinia,* and *Listeria*, likely because these organisms can be genetically manipulated with relative ease (Cossart *et al.*, 2003;Finlay and Cossart, 1997;Gruenheid and Finlay, 2003;Lesser *et al.*, 2000;Patel and Galan, 2005). While there are some commonalities in the processes employed by these different organisms to trigger their own uptake into non-phagocytic cells, each pathogen does utilize unique processes during invasion.

Modulation of host cell calcium and/or phosphoinositides often regulates or modulates actin binding proteins in the host cell. These moieties may be recruited to the site of the organism's entry to participate in the host cell cytoskeletal modification and actin remodeling that occurs during invasion. In mammalian cells, the major actin rearrangements that occur upon stimulation are membrane ruffling, filopodia formation, and actin stress fiber formation (Hall, 1998;Tapon and Hall, 1997). These rearrangements can be mediated by members of the Rho-family GTPases (Rac, Rho, and Cdc42)---GTPases that have been previously demonstrated to play a role in the invasion process of some bacterial pathogens (Boquet and Lemichez, 2003;Guzman-Verri *et al.*, 2001;Lesser *et al.*, 2000;Verma *et al.*, 2000).

The host cell invasion process and corresponding morphological changes induced at the host cell surface by an invading pathogen have been broadly characterized into two categories—the "zipper" and the "trigger" mechanisms of invasion (reviewed in Finlay and Cossart, 1997). In "zipper" type invasion, attachment to the host cell is mediated by direct contact between a ligand on the surface of the pathogen and a host cell receptor. This leads to activation of host cell receptor tyrosine kinases and a cascade of signal transduction events in the host cell. This is followed by localized cytoskeletal rearrangements and a tight "zippering" of the host cell membrane around the bacterium as it enters the host cell. *Yersinia* is one of the best studied zipper-type invasive pathogens; the *Yersinia* outer membrane protein invasin mediates attachment and triggers the host cell signal transduction cascade leading to epithelial cell invasion of this organism (Finlay and Cossart, 1997;Gruenheid and Finlay, 2003;Isberg *et al.*, 2000).

In contrast to the subtle, localized, cytoskeletal modifications induced by zipper-type pathogens, organisms employing trigger-type invasion inject bacterial proteins directly into the host cell cytoplasm resulting in dramatic cytoskeletal rearrangements in the host cell, such as membrane ruffles and large membrane projections (Finlay and Cossart, 1997). *Salmonella* is one example of a well-studied trigger type pathogen. *Salmonella* triggers invasion by injecting bacterial effector proteins into the host cell by way of a type III secretion system resulting in modification of host cell signal transduction pathways, such as activating the Rho-family GTPases Rac1 and Cdc42, to facilitate *Salmonella* uptake into non-phagocytic host cells (Finlay and Cossart, 1997;Galan and Zhou, 2000;Patel and Galan, 2005).

Once inside the host cell, various fates for the bacterium exist. In some cases, the host cell phagosome will mature into a lysosome and, in order to survive, some pathogens have adapted strategies to persist in this niche (Finlay and Cossart, 1997;Gruenheid and Finlay, 2003). Some organisms have evolved with mechanisms to arrest phagosome maturation and prevent lysosome formation, and some organisms possess the means to escape from the host vacuole into the host cell cytosol (Finlay and Cossart, 1997). There are also various fates for the host cell infected with an invasive bacterium. For example, some pathogens manipulate the programmed cell death process of the host cell. One strategy is to delay apoptosis in order to maintain a replicative niche for the organism, while other pathogens induce apoptosis to facilitate bacterial spreading and breach of host barriers (Gao and Abu, 2000;Gao and Kwaik, 2000).

The ability to invade host cells presents numerous advantages to a bacterial pathogen such as the ability to hide from the host's immune system, enter a protected niche, and the ability to gain access to privileged host sites (Gao and

Abu, 2000;Gao and Kwaik, 2000). Thus, it is not surprising that so many bacterial pathogens have evolved mechanisms to invade a variety of host cell types.

1.3. Surface associated glycolytic enzymes in bacterial pathogenesis

Most metabolic enzymes, such as those that participate in the glycolytic pathway, are localized to the cellular cytoplasm. Historically, these enzymes have been categorized as "housekeeping" enzymes, and for many years did not garner much interest in investigations on pathogenesis in eukaryotes or prokaryotes. However, there have been a number of studies in recent years revealing that glycolytic enzymes have a much broader repertoire of functions than was previously assumed. For example, in eukaryotic cells, glycolytic enzymes have been demonstrated to play a role in transcriptional regulation, maintenance of glucose homeostasis, regulation of apoptosis, and cell motility (reviewed in Kim and Dang, 2005).

In addition to the multifunctional role glycolytic enzymes can play in eukaryotes, a large number of such enzymes have been found on the surface of a variety of organisms pathogenic to humans. This theme transcends kingdoms and is observed in bacteria, fungi, and parasites. One example in Streptococci is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was reported on the Group A Streptococcal (GAS) cell surface at the same time by two groups, as well as on the surface of *Streptococcus pneumoniae* (Bergmann *et al.*, 2004;Pancholi and Fischetti, 1992;Winram and Lottenberg, 1996), and GBS (Hughes *et al.*, 2002;Seifert *et al.*, 2003). A variety of functions for GAPDH on the surface of GAS (termed surface dehydrogenase, SDH), have been characterized in addition to its innate glycolytic activities, including binding to mammalian proteins (such as plasminogen, fibronectin and myosin), ADP ribosylation of host cell proteins, condensation of host cell DNA (an event suggestive of induction of apoptosis), and manipulation of host cell signaling pathways, including modulation of host cell tyrosine kinases and serine/threonine kinases (Boel *et al.*, 2005;Pancholi and Fischetti, 1992;Pancholi and Fischetti, 1993;Pancholi and Fischetti, 1997a;Pancholi and Fischetti, 1997b). The multifunctionality of surface associated GAPDH has also been demonstrated in *S. pneumoniae*; it has been found that pneumococcal GAPDH binds to plasminogen, is immunogenic, and that pneumococcal strains resistant to macrolide antibiotics have more GADPH on their surface compared to sensitive strains, suggesting this glycolytic enzyme plays a role in virulence of *S. pneumoniae* (Bergmann *et al.*, 2004;Cash *et al.*, 1999;Ling *et al.*, 2004). GAPDH on the surface of GBS (Hughes *et al.*, 2002;Seifert *et al.*, 2003) has been found to interact with cytoskeletal and extracellular matrix proteins including plasminogen, actin, and fibrinogen (Seifert *et al.*, 2003).

GAPDH is also found on the surface of other pathogens such as *Staphylocococcus aureus* (Modun and Williams, 1999), *Neisseria meningiditis* (Grifantini *et al.*, 2002), *Candida albicans* (Alloush *et al.*, 1997), *Saccharomyces cerevisiae* (Delgado *et al.*, 2001), and *Schistosoma mansoni* (Argiro *et al.*, 2000). Another example, α-enolase, has been found on the surface of Streptococci

(Bergmann *et al.*, 2001;Lottenberg *et al.*, 1992;Pancholi and Fischetti, 1998;Winram and Lottenberg, 1996), *C. albicans* (Angiolella *et al.*, 1996;Crowe *et al.*, 2003), and *Pneumocystis carinii* (Fox and Smulian, 2001). The fact that these glycolytic enzymes, proteins that are typically thought of as being localized to the cytoplasm, are found on the surface of so many pathogens (a location unrelated to their obvious function) suggests that these moieties also possess alternative functions, and may participate in the pathogenic process.

Movement of glycolytic enzymes from the cytoplasm to the cell surface is necessary for interactions between bacterial proteins and the host cell to occur. For Gram-positive bacteria, the cell wall functions as an "organelle" of sorts to anchor and display molecules on the cell surface, many of which can act as adhesive molecules. Several mechanisms to direct Gram-positive proteins to the cell surface have been characterized. The most common mechanism is for a putative surface protein to be synthesized as a precursor with an N-terminal signal peptide and a C-terminal sorting signal (which is comprised of an LPXTG motif, a hydrophobic domain, and a tail of positively charged residues) (Scott and Barnett, 2006;Ton-That *et al.*, 2004). Once the protein is directed to the surface of the cell, the signal sequence is cleaved and the resulting peptide becomes a substrate for cleavage by sortase (Scott and Barnett, 2006;Ton-That *et al.*, 2004). This is followed by linkage of the protein to the cell wall by transglycosolyation or transpeptidation reactions. Other mechanisms for Gram-positive surface protein localization/anchoring include binding of proteins to teichoic or lipoteicoic acids, insertion of the protein into the cell membrane via an anchor structure or N-terminal diacylglycerol, and binding to the cell wall or membrane by charge or hydrophobic interactions (Scott and Barnett, 2006;Ton-That *et al.*, 2004). However, a common feature of the glycolytic enzymes displayed on the cell surface of Streptococci and other Gram-positive organisms is that they possess no known mechanism for sorting or attachment to the cell surface.

There are two prevailing schools of thought regarding how Gram-positive glycolytic enzymes come to reside on the outer surface of the organism for display to the surrounding milieu. One theory is that these enzymes are released from the bacteria into the environment in a passive process (possibly leakage from dead cells) and then become re-associated with the organism by binding to a receptor on the bacterial cell surface (Chhatwal, 2002), although experimental evidence for this theory is lacking. The other possibility is that these proteins are directed to the cell surface by a novel sorting or anchoring mechanism yet to be discovered. In mutational studies of GAS SDH in which the C-terminus was modified with a hydrophobic peptide, the protein was sequestered inside the bacterial cell, and no SDH was found on the surface (Boel *et al.*, 2005). If SDH display on GAS surface was a passive process secondary to leaked SDH, or a result of SDH released from lysed cells into the culture medium, one would expect equivalent amounts of SDH located on both mutant and wild-type GAS.

"Enzymatic proteins" that have been described on the GBS cell surface include GAPDH, enolase, ornithine carbamolytransferase, purine nucleoside phosphorylase, glucose-6-phosphate isomerase, glutamine synthase, and phosphoglycerate kinase (Hughes *et al.*, 2002;Madureira *et al.*, 2007;Seifert *et al.*, 2003).

The discovery that the surface-associated glycolytic enzyme phosphoglycerate kinase (PGK) is a virulence factor for GBS and characterization of role that PGK plays in GBS pathogenesis, is a major focus of this thesis and is described in detail in Chapters 3 and 4.

1.4. Thesis overview and objectives

Group B *Streptococcus* emerged as a major human pathogen in the 1970s. Since then, there have been many epidemiological studies on this organism, with a focus on identification of individuals at risk, preventative therapies, and treatment of individuals who acquire GBS disease. As a result of these investigations and the guidelines regarding GBS issued by the CDC in 1996 and 2002, a marked decrease in the incidence of GBS disease and case fatality rates in neonates and pregnant women has been observed. There is, however, a certain residual burden of neonatal GBS disease that is not addressed by chemoprophylaxis alone and further, these guidelines do not address the problem of invasive GBS infections in non-pregnant adults.

In the last ten years, the focus of investigations on GBS has shifted from mainly clinical and epidemiological studies to the examination of the genetics and virulence factors of this organism that contribute to its ability to cause invasive disease. A number of GBS virulence factors have been identified and characterized, and it has been clearly demonstrated that GBS are able to invade a number of host cell types, including epithelial cells, endothelial cells, and macrophages (Gibson *et al.*, 1993;Greco *et al.*, 1995;Lalonde *et al.*, 2000;Nizet *et al.*, 1997;Shin and Kim, 2006;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1996;Valentin-Weigand *et al.*, 1997). The fact that GBS has the ability to bind to and invade a variety of host cell types suggests that either this organism interacts with a widely distributed host cell moiety to facilitate this process, or that it uses a combination of processes to bind to and interact with factors in/on host cells.

The attachment and invasion of GBS into host cells is thought to involve numerous host-pathogen interactions. At the commencement of the work contained in this thesis, an understanding of these events at the molecular level was only in the early stages of characterization. What was clearly established is that host cell actin microfilaments are recruited to the site of GBS attachment and invasion, and that modulation of actin microfilaments by GBS is essential for invasion to occur. This was demonstrated by the finding that the addition of cytochalasin D (a potent inhibitor of actin polymerization) to epithelial cells prior to infection inhibits GBS invasion (Gibson *et al.*, 1993;Greco *et al.*, 1995;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997), as well as microscopic demonstration of actin recruitment to the site of GBS attachment (Fig. 1.5) (Tyrrell *et al.*, 2002).

The epithelium is a key component of innate immunity for humans. Breech of the epithelial barrier by a bacterial pathogen is often the precipitating event in invasive disease. Although in some situations GBS causes asymptomatic colonization of the host, the ability of GBS to invade epithelial cells is central to the ability of the organism to cause invasive disease, a process contributing to the clinical features of GBS infection. Thus, the broad objective of my thesis work was to characterize the process of GBS invasion into epithelial cells at the molecular level, with a focus on actin manipulation and recruitment by this organism. Investigations targeted at the elucidation of processes occurring at the interface between GBS and the host is an important avenue towards the development of novel therapeutic agents, and identification of bacterial surface proteins involved in invasion or host-cell actin manipulation present enticing vaccine targets. Thus, this thesis is focused on examining host epithelial cell signal transduction processes involved in the GBS invasion process, and phosphoglycerate kinase, a GBS surface protein that interacts with host cell actin.

Table 1.1. Summary of the virulence factors of Group B *Streptococcus*.

Virulence Factor	**Known or proposed function in virulence**
Capsule	Evasion of opsonophagocytosis by the host's immune system
β-hemolysin	Pore-forming, non-immunogenic cytolysin that is active against many host cell types
CAMP factor	May be be a pore forming toxin Results in the characteristic CAMP reaction of GBS
Lipoteichoic acid	May mediate bacterial adherence to eukaryotic cells
Hyaluronate Lyase	May be a spreading factor to facilitate dissemination through the connective tissue matrix of the host
C Protein	Alpha antigen may facilitate immune evasion by antigenic variation Host cell adherence and invasion β antigen possesses IgA binding domain
Superoxide dismutase	Combat oxidative stress by converting superoxide anions to less toxic substances
C5a Peptidase	Cleaves and inactivates the neutrophil chemotaxin C5a Binds to fibronectin
Interactions with extracellular matrix proteins	Adhesion to these components may contribute to colonization of mucosal and epithelial surfaces
Pili	May facilitate adhesion and invasion into host cells

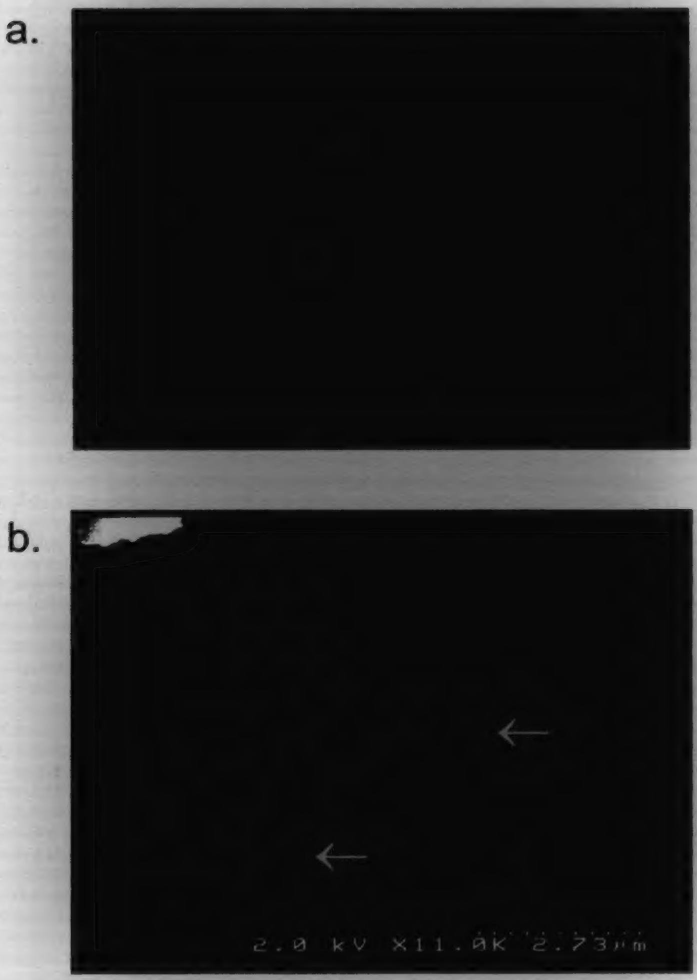

Fig. 1.1. The Group B Streptococcus. (a). Fluorescence image of long chains of GBS grown in broth culture. (b). Electron micrograph showing chains of GBS (pink, arrows) interacting with the surface of a HeLa cell. Tyrrell Laboratory photos.

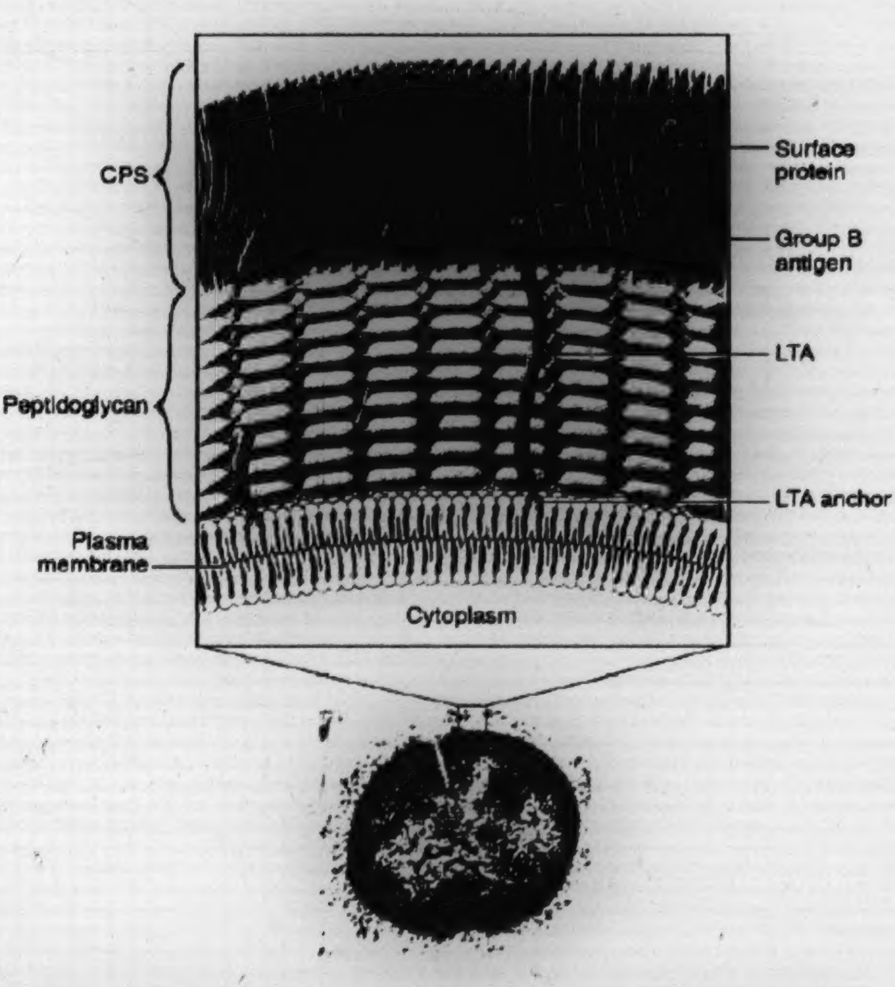
CPS
Peptidoglycan
Plasma membrane
Surface protein
Group B antigen
LTA
LTA anchor
Cytoplasm

Fig. 1.2. Schematic of the cell wall structure of GBS. An electron micrograph of a serotype III GBS is shown below a cartoon illustrating important surface structures of this organism. The GBS cytoplasm is bound by a cell membrane that is surrounded by a peptidoglycan layer. Anchored in the peptidoglycan layer is the capsular polysaccharide (CPS) and the group B antigen. Lipoproteins and glycolipids extend out from the cell membrane. Lipoteichoic acid (LTA) is also anchored into the membrane. Many surface proteins attach to the peptidoglycan. (Adapted from Baron and Kasper,2005).

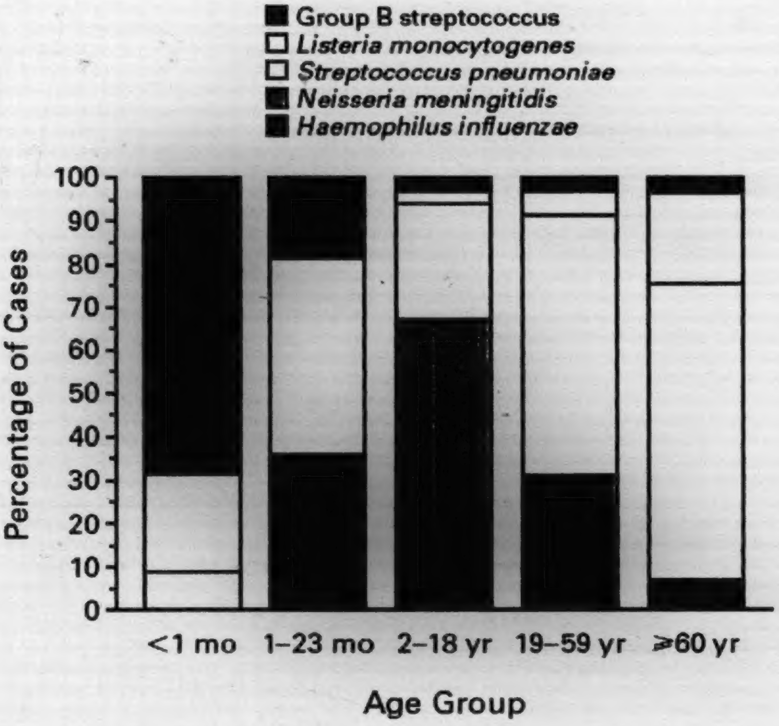

Fig. 1.3. Proportion of cases of meningitis caused by bacterial pathogens in different age groups, excluding meningitis caused by enteric pathogens such as *E. coli*. Approximately 70% of the meningitis cases in individuals less than one month of age are due to GBS. (Adapted from Schuchat *et al.*,1997).

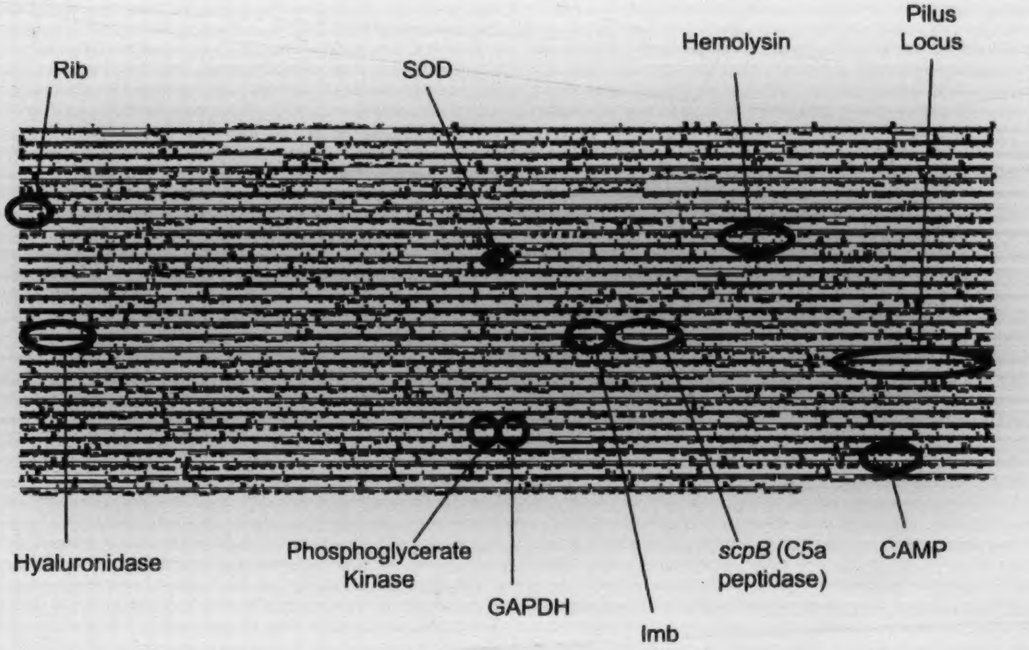
Rib
SOD
Hemolysin
Pilus Locus
Hyaluronidase
Phosphoglycerate Kinase
GAPDH
lmb
scpB (C5a peptidase)
CAMP

Fig. 1.4. Linear representation of a serotype V GBS genome. The location of some genes related to virulence are highlighted. (Adapted from Tettelin et al.,2002.).

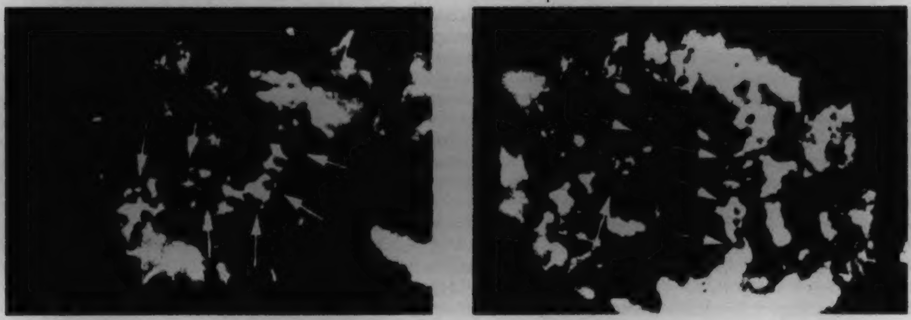

Fig. 1.5. Actin is recruited to the site of GBS attachment and invasion into HeLa cells. HeLa cells infected with GBS for 1 h were fixed, permeabilized, and stained with FITC-conjugated phalloidin to label filamentous actin. Cells were visualized using fluorescence microscopy. Fluorescently stained actin is observed around the perimeter of the chains of streptococci, as indicated by the arrows. (Adapted from Tyrrell *et al.*,2002).

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Chapter 2:

Quantitation and Live Microscopic Analysis of the Invasion of Group B *Streptococcus* into Cultured Human Cell Lines

2.1. Introduction

The Group B *Streptococcus* (GBS) is a Gram-positive, opportunistic human pathogen. Although in some situations GBS colonizes the host asymptomatically, the ability of GBS to invade epithelial cells is central to the ability of the organism to cause invasive disease, a process contributing to the clinical features of GBS infection. GBS is widely recognized for its ability to cause severe disease in neonates, such as pneumonia and septicemia, and is the most common causative agent of meningitis for infants less than one month of age in the Western world (Puopolo *et al.*, 2005;Schuchat *et al.*, 1997;Schuchat, 1998). Disease caused by GBS is not restricted to infancy but can also occur in adulthood, both in post-partum women and in the elderly, especially in those with an underlying chronic illness, such as diabetes mellitus (Farley *et al.*, 1993;Schuchat *et al.*, 1990;Schuchat, 1998;Tyrrell *et al.*, 2000). The most commonly identified GBS serotype both colonizing and causing invasive disease in older adults is serotype V (Edwards *et al.*, 2005).

Although for many years Streptococci were considered to be exclusively extracellular pathogens, it has now been well documented that GBS are able to invade numerous host cell types, including epithelial cells, endothelial cells, and macrophages (Gibson *et al.*, 1993;Lalonde *et al.*, 2000;Nizet *et al.*, 1997;Shin and Kim, 2006;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1996;Valentin-Weigand *et al.*, 1997). The entry of a bacterial pathogen into non-phagocytic host cells is a complex process, usually involving multiple bacterial and host cell factors. A common theme in bacterial invasion of host cells is modulation and exploitation of the host cell cytoskeleton, leading to transient changes in cell architecture to facilitate bacterial uptake. This is true of GBS invasion. Our laboratory and others have demonstrated that the addition of cytochalasin D, a potent inhibitor of actin polymerization, inhibits GBS invasion into epithelial cells (Gibson *et al.*, 1993;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997) and that actin is recruited to the site of GBS attachment and subsequent invasion (Tyrrell *et al.*, 2002). While several GBS virulence factors have been described (reviewed in Burnham and Tyrrell, 2003;Doran and Nizet, 2004), the specific molecular processes contributing to actin recruitment and host-cell signal transduction processes involved in cytoskeletal rearrangements during the GBS epithelial cell invasion process are yet to be fully characterized.

The objective of this chapter was to characterize the invasive characteristics of a serotype V GBS strain, NCS13, by examining its ability to attach to and invade cultured human cell lines.

2.2. Materials and Methods

2.2.1. Bacterial strains and growth conditions.

The GBS and *Escherichia coli* strains used in this study are listed in Table 2.1. The organisms were cultured at 35 °C on sheep blood agar plates (BAPs)

(Dalynn Biologicals) or in Todd-Hewitt Broth (THB) (Difco Laboratories). Broth cultures were grown with shaking when required.

2.2.2. Cell lines and growth conditions.

The human epithelial cell line HeLa 229 (ATCC CCL-2.1) (cervical epithelial tumor cell line) was grown and maintained in OPTI-MEM I Reduced Serum Medium (Invitrogen) with 4% fetal bovine serum (FBS) (Gibco BRL) unless otherwise noted. The human epithelial cell lines HEp-2 (ATCC CCL-23) (cervical epithelial tumor cell line) and A549 (ATCC CCL-185) (lung epithelial tumor cell line) were grown in Minimal Essential Medium (MEM) supplemented with 10% FBS and 1% glutamine. The fibroblastic cell line MRC-5 (ATCC CCL-171) (normal lung cell line from a 14 week old fetus) was also grown in MEM with 10% FBS and 1% glutamine. All cell lines were obtained from the American Type Culture Collection (ATCC).

2.2.3. Antibiotic protection (invasion) assay.

Invasion of bacteria into cultured cell monolayers was quantified using a standard antibiotic protection invasion assay with minor modifications (Rubens *et al.*, 1992;Tyrrell *et al.*, 2002). Briefly, eukaryotic cells were grown to confluence in 24 well plates (Corning). GBS or DH5α were grown overnight in THB at 35 °C with shaking. The next day, the overnight growth was adjusted to a 0.5 McFarland standard (approximately 1.5 X 10^8 colony forming units per mL), and then diluted

1 in 100 in THB. One hundred μL of this dilution (approximately 1.5 X 10^5 organisms) were used to infect each well. The 1 in 100 dilution of the 0.5 McFarland standard was diluted further 1 in 100 for input colony counts, which were performed in triplicate.

Following inoculation, the cell culture plates were centrifuged at 100 *g* for 5 min at room temperature, and then incubated at 35 °C for 2 h to allow bacterial internalization to occur. Portions of HeLa cell supernatants were plated onto BAPs following the initial 2 h infection period to confirm the purity and viability of the inoculum in each well. The monolayers were then washed three times with phosphate-buffered saline (PBS) to remove unbound bacteria. Bacteria that had bound but not internalized were killed by incubation for 2 h with fresh media containing 5 μg of penicillin per mL and 100 μg of gentamicin per mL. After the 2 h incubation, an aliquot of media from each well was applied to a BAP to ensure that the antibiotic treatment was adequate to kill all extracellular organisms. The cell monolayers were then washed with PBS, trypsinized and then lysed with 0.1% Triton X-100, the lysates spread onto BAPs, and incubated overnight at 35 °C. The number of colonies on each plate was counted to assess the number of CFU that had invaded the monolayer.

2.2.4. Attachment assay.

To visually examine adhesion of NCS13 to various cell lines, HeLa, A549, or MRC-5 cells were grown on sterile glass coverslips in shell vials. Overnight growth of GBS in THB was adjusted to a 0.5 McFarland standard and then diluted 1 in 100 in THB. Fifty μL of this suspension (corresponding to approximately 7.5 X 10^4 CFU) was added to the cell monolayer. The shell vials were centrifuged at 100 *g* for 5 min at room temperature, and then incubated at 35 °C for 1 h or 2 h. At the appropriate time points, the coverslips were washed 3 times with PBS and then fixed in 3% formaldehyde-PBS for 30 min at room temperature. The coverslips were mounted onto glass slides and examined using phase contrast microscopy.

2.2.5. Transient transfection of HeLa cells.

Transfection of HeLa cells with the vector pEGFP-actin (Clontech) was performed using Lipofectamine 2000™ Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, HeLa cells were grown in 3 cm Glass Bottom Culture Dishes (MatTek Corporation). When the cell density reached approximately 80% confluence, the culture media was replaced with Opti-MEM I (no FBS), and the cells were transfected with a Lipofectamine:DNA complex, with each transfection reaction containing 1 μg of plasmid DNA and 2 μg of Lipofectamine. Following transfection, the cells were incubated for 4 hours at 37 °C, and the media replaced with fresh Opti-MEM I with 4% FBS. The cells were incubated overnight at 37 °C (approximately 18-22 h) and analyzed using confocal microscopy the following day.

2.2.6. Confocal microscopy.

For live microscopic analysis of GBS infecting host cells, the MatTek Glass Bottom Culture dish containing HeLa cells transfected with pEGFP-actin was placed in a microscope stage warmer set to 37 °C. Two μg of Hoechst dye (Invitrogen) was added to the dish, and then the cells were challenged with an inoculum of 3.0 X 10^7 CFU NCS13. A Carl Zeiss Laser Scanning 510 Confocal Microscope was used to image the interaction of GBS with the cells using a 40X objective. An ultra-violet laser with 364 nm wavelength was used to visualize Hoechst staining and an Argon laser with a 488 nm wavelength was used to visualize GFP-actin. Images were captured using the software program Metamorph (Universal Imaging Corp.) and analyzed further using the Zeiss LSM 5 image browser.

2.2.7. Fibronectin "inhibition" assay.

For the fibronectin inhibition assay, an antibiotic protection invasion assay was performed with slight modifications. NCS13 was grown overnight in THB with shaking. This overnight growth was adjusted to a 0.5 McFarland standard in THB, and then diluted 1 in 100 in THB. The NCS13 were either left untreated, or a solution of fibronectin from bovine plasma was added to aliquots of NCS13 to final concentrations of 0.1, 1, 10, and 100 μg/mL. The NCS13-GBS/fibronectin mixtures were incubated with gentle agitation at 35 °C for 30 min, and then 50 μL of the solution (corresponding to approximately 7.5 X 10^4 CFU) was used to inoculate HeLa or MRC-5 cells. The invasion assay procedure described in Section 2.2.3 was followed for the remainder of the assay. Portions of the HeLa cell culture supernatants were plated on to BAPs following the initial 2 h infection period to ensure that the fibronectin treatment did not alter bacterial viability. The relative percent invasion was calculated as follows: [(Number of fibronectin treated GBS CFU invaded/Number of untreated GBS CFU invaded) X 100%].

2.3. Results

2.3.1. Quantitation of NCS13 invasion into human cell lines.

To quantitate invasion of the GBS strain NCS13 into cultured human cell lines, an antibiotic protection invasion assay was performed. NCS13 invade HeLa cells with the highest rate of efficiency (approximately 3.5% invasion), followed by HEp-2 cells with an average rate of 2.5% invasion, and A549 lung cells with an average of 1.5% (Fig. 2.1). NCS13 invaded the fibroblastic cell line MRC-5 very poorly, with an average invasion rate of 0.1% (Fig. 2.1). The invasion rates depicted in Fig. 2.1 are indicative of one representative experiment with typical results, however intra-assay variation in invasion was observed, with HeLa cell invasion ranging from 2 to 4%, HEp-2 cell invasion ranging from 2 to 3%, and A549 invasion ranging from 1 to 2% of the initial NCS13 inoculum. The rate of MRC-5 invasion was never found to be greater than 0.2%.

2.3.2. NCS13 attachment to human cell lines.

The relative amount of NCS13 attachment to host cell lines was examined using phase contrast microscopy to determine if efficiency of invasion correlated with efficiency of bacterial attachment to the cell lines assayed, or if attachment without subsequent invasion occurs. HeLa, A549 and MRC-5 cells were infected with NCS13 for 1 h or 2 h, and visualized using phase contrast microscopy.

Many NCS13 chains were attached to HeLa cells at 1 h post infection, and by 2 h the HeLa cells were nearly covered with chains of streptococci (Fig. 2.2a). Fewer NCS13 bound to A549 cells at 1 h than bound to HeLa cells, but by 2 h post-infection, large numbers of NCS13 were attached to A549 cells also (Fig. 2.2b). In contrast, very few NCS13 chains attached to MRC-5 cells, even at 2 h post-infection (Fig. 2.2c).

2.3.3. GBS strains vary in human epithelial cell invasion efficiency.

As the invasion of GBS serotype V isolates is not well characterized in the literature, the invasion of NCS13 was compared to other serotype V strains. BAA-611 is a serotype V clinical isolate whose genome has been sequenced in entirety (Tettelin *et al.*, 2002). In contrast to NCS13, BAA-611 (also known as 2603 V/R) invaded HeLa and A549 cells very poorly, and invaded HEp-2 cells with the highest efficiency (Fig. 2.3). However, the rate of HEp-2 cell invasion by BAA-611 is only 0.5%, lower than the 2.5% invasion observed with NCS13. GBS strain SS1169 (also known as ATCC 49447) is one of the first serotype V strains described in the literature (Jelinkova and Motlova, 1985). Its invasion profile closely matches that of BAA-611 (Fig. 2.3). In addition, two clinical GBS isolates from our laboratory's collection, GBS3855 and GBS4030, were also assayed for epithelial cell invasion. These two strains invaded HEp-2 cells as efficiently as NCS13 (approximately 2.5% of the inoculum) (Fig. 2.3). However, HeLa and A549 cells were invaded very poorly by GBS3855 and GBS4030. *E. coli* DH5α, the negative control for invasion, did not invade any of the cell lines assayed (Fig. 2.3).

2.3.4. Live microscopic analysis of NCS13 invasion of HeLa cells.

HeLa cells were transfected with a plasmid (pEGFP-actin) expressing GFP-actin, infected with NCS13, and visualized using confocal microscopy. Initially, the intent was to capture recruitment of actin microfilaments to the site of GBS attachment and invasion in real-time in live cells. It was expected that this would be a transient, localized event and thus images were captured every 15 s. However, phototoxicity of the host cells was observed in response to this continuous, high-intensity laser treatment. Over the course of the analysis, many of the HeLa cells began to show signs of apoptosis. An example of this was captured and is shown in Fig. 2.4. Further, it became obvious that the level of magnification that would be required to adequately resolve actin microfilament rearrangement would result in even higher level laser intensity to the live cells, and would not be a viable approach. Thus, I shifted my focus to examining the general invasion of NCS13 into live cells in real time. Captured images can be compiled into a short movie to visualize the invasion process, or images from individual time points can be examined.

Figures 2.5a and 2.5b illustrate NCS13 invading HeLa cells over 70 min. At 14 min (840 s) post infection, most of the HeLa cells had at least one chain of NCS13 attaching to and/or invading the cell. By 70 min (4200 s), the final time point assayed, many of the HeLa cells were being invaded by multiple chains of NCS13.

These images suggest that NCS13 attach to and invade HeLa cells in a polar fashion, i.e. one end of the chain enters the host cell first, and the rest of the chain subsequently follows, rather than the whole chain attaching along the length and entering the cell from the "side".

Images of NCS13 infecting HeLa cells were also captured through the Z-plane of the cells. This is akin to focusing up and down on the specimen with the microscope. By capturing thin slices (0.11 μM) NCS13 were found in the same plane as the HeLa cells, confirming that NCS13 invaded these cells and that the GBS chains were not just adhering to the HeLa cell surface (Fig. 2.5c).

2.3.5. Incubation of NCS13 with soluble fibronectin prior to infection enhances HeLa and A549 cell invasion.

It has been hypothesized that GBS invasion may be facilitated by bacterial binding to fibronectin on the host cell surface. To explore this, I incubated NCS13 with soluble fibronectin for 30 min prior to infection of HeLa, A549, and MRC-5 cells. Presumably, if GBS bind to fibronectin on the host cell surface this pre-incubation step would saturate fibronectin binding sites, and reduce GBS invasion. What was observed, was that HeLa and A549 cell invasion by NCS13 is *increased* as a result of the pre-incubation step with fibronectin. A 60% increase was observed at the low concentration of 0.1 μg of fibronectin per mL in HeLa cells, but appeared to saturate quickly and did not increase further at 1, 10, or 100 μg per mL (Fig. 2.6a). In A549 cells, no modification in invasion was observed at 0.1 μg per mL of fibronectin, but invasion was increased by 30 to 40% with 1, 10, and 100 μg per mL fibronectin (Fig. 2.6b). In contrast to the effect seen with HeLa cells, pre-incubation of NCS13 with soluble fibronectin did not enhance invasion into MRC-5 cells. MRC-5 cell invasion by GBS did not exceed 0.15% at any fibronectin concentration assayed (Fig. 2.6c).

2.4. Discussion

It is thought that the ability of GBS to invade host cells is central to the disease manifestations caused by this organism, such as meningitis and septicemia. Invasion of host cells by a bacterial pathogen poses several advantages to the bacterium; for example it can facilitate evasion of the host immune system and promote access to privileged sites in the human body. In this chapter, I sought to characterize the ability of a serotype V GBS strain, NCS13, to invade cultured human cells with the goal of using that data as a tool for future study of GBS pathogenesis and events at the GBS-host cell interface.

I observed that NCS13 invaded HeLa, A549, and HEp-2 cells efficiently; all of these cells are epithelial in nature. In contrast, NCS13 invaded the fibroblastic cell line MRC-5 very poorly. The ability of NCS13 to invade the cultured epithelial cells was several fold higher than the other GBS strains assayed, including the GBS strain whose genome has been entirely sequenced (Tettelin *et al.*, 2002).

Great genetic diversity exists between different GBS strains. Tettelin and colleagues sequenced a collection of GBS strains from all nine known serotype designations, in an attempt to quantify the "core genome" of GBS (Tettelin *et al.*, 2005). However, their analysis revealed that there is a continuum of diversity amongst GBS isolates, and while approximately 1800 genes are found in every strain, an average of 33 unique genes are identified in each new strain that is sequenced (Tettelin *et al.*, 2005). While this analysis helped establish that the current system of serotype designation does not reflect genetic diversity or invasive potential between strains, it is not yet clear is if these "fringe" genes are what is responsible for or contribute to the difference in invasive potential that exists between strains.

While many of the molecular aspects of GBS invasion are not fully characterized, it is known that actin microfilaments are involved in the invasion process (Greco *et al.*, 1995;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997). I sought to use confocal microscopy of live HeLa cells transfected with GFP-actin to explore the actin recruitment process in real-time with the intent of determining at what time point actin is recruited during GBS invasion and how long transient rearrangement of the host cell cytoskeleton can be observed during this process. I faced technical limitations during this analysis as repeated exposure to the level of laser intensity required to capture the detail required was toxic to the HeLa cells. However, I was able to capture images of live GBS invading live HeLa cells in real time; to my knowledge, this is the first time this type of analysis has been performed for this species. I observed that GBS invade HeLa cells quickly after infection, that multiple GBS chains can infect a single cell, and that GBS invade in a polar fashion. This analysis provided real-time evidence of GBS invasion of epithelial cells.

In the human body, fibronectin is found in two forms—a soluble form found in plasma, and an insoluble, tissue associated form. While it has been shown with a relative degree of certainty that GBS have the ability to bind several extracellular matrix components in purified form, such as cytokeratin (Tamura and Nittayajarn, 2000) and lamin (Spellerberg *et al.*, 1999), studies into the ability of GBS to bind to soluble fibronectin are less clear, and certainly more controversial. In 1987, Butler and colleagues found that GBS bound fibronectin purified from human plasma at only a very low level, which they deemed to be insignificant (Butler *et al.*, 1987). However, later investigations found that GBS immobilized to a polystyrene dish bound to bovine fibronectin, and the binding curve was very sensitive, dose-dependant, and became saturated at a fibronectin concentration of 20 μg/mL (Rainard, 1993). In 1995, Tamura and Rubens observed that GBS adhere to fibronectin immobilized onto a polystyrene microtitre plate (Tamura and Rubens, 1995). Thus, it is possible the amount of fibronectin binding first considered to be "insignificant" actually represents a phenomenon that is biologically significant.

Fibronectin participates in the surface colonization and invasion of several bacterial pathogens, including some Streptococcal species. For example, with GAS, fibronectin acts as a "bridge" molecule to connect protein F1 on the GAS surface to integrin receptors on the host cell with subsequent bacterial uptake (Ozeri *et al.*, 1998). Ozeri *et al.* observed that the addition of soluble fibronectin to the cell culture medium increases GAS invasion of HeLa cells; this increase is observed at the very low fibronectin concentration of 0.1 μg per mL but the effect becomes quickly saturated and plateaus at 1 μg of fibronectin per mL, illustrating that a small change in fibronectin concentration results in a large alteration in GAS entry (Ozeri *et al.*, 1998).

In conjunction with my investigations into the host cell invasion process by GBS, I sought to explore if the GBS interaction with fibronectin is an essential component of the invasion process. I found that while incubation of GBS with soluble fibronectin prior to host cell infection enhances GBS invasion of HeLa cells and A549 cells, it does not alter the ability of GBS to invade MRC-5 cells. This suggests that while fibronectin may augment the ability of GBS to invade some host cell types, fibronectin alone is not sufficient to confer host cell entry. That is, the addition of fibronectin to GBS does not alter the organism such that it becomes able to invade a cell type that it does not invade efficiently in the absence of fibronectin. Further to this, previous investigations have illustrated that MRC-5 cells possess 2.7 fold more fibronectin on their surface than HeLa cells (Tyrrell *et al.*, 2002). Thus, fibronectin on the host cell surface appears to be insufficient to confer GBS internalization. These data together suggest a scenario for fibronectin mediated GBS invasion similar to what occurs with GAS, whereby fibronectin acts as a bridging molecule, bringing together an adhesion on the bacterial surface with a receptor on the host cell.

C5a is a potent chemotaxin that facilitates recruitment of neutrophils to sites of inflammation; GBS possess C5a peptidase on their surface which is capable of inactivating C5a. During the time my GBS-fibronectin invasion studies were being performed, two separate groups published data illustrating that C5a peptidase is a bifunctional GBS surface protein; in addition to its peptidase function, it is also a fibronectin binding protein (Beckmann *et al.*, 2002;Cheng *et al.*, 2002). One of these groups, Cheng and colleagues, also observed that the addition of soluble fibronectin to the cell culture medium increases GBS invasion into A549 cells (by approximately 20%) but that GBS invasion into HEp-2 cells is not altered by this treatment (Cheng *et al.*, 2002). This data suggests that GBS invasion of host cells involves multiple bacterial and host cell factors, and that different processes are involved in different host cell types.

In conclusion, I have demonstrated that a serotype V GBS strain, NCS13, invades HeLa, HEp-2, and A549 cells. Invasion of NCS13 into cultured human cell lines provided me with a tool for future analysis to examine and characterize GBS-host cell interactions and the specifics of the invasion process.

Table 2.1. Bacterial strains used in this investigation.

Strain	Relevant Description	Reference or Source
GBS		
NCS13	Serotype V; isolated from the soft tissue wound of an elderly patient.	(Tyrrell *et al.*, 2002)
BAA-611	Serotype V; complete genome is sequenced. Also referred to as 2603 V/R.	ATCC; (Tettelin *et al.*, 2002)
SS1169	First serotype V strain deposited with ATCC; also referred to as ATCC 49447.	ATCC; (Jelinkova and Motlova, 1985)
GBS3855	Clinical vaginal specimen from a 26 year old female.	Our laboratory collection
GBS4030	Clinical swab specimen from a 7 month old male.	Our laboratory collection
E. coli		
DH5α	High efficiency cloning strain, does not invade host cell lines.	Our laboratory stocks; (Woodcock *et al.*, 1989)

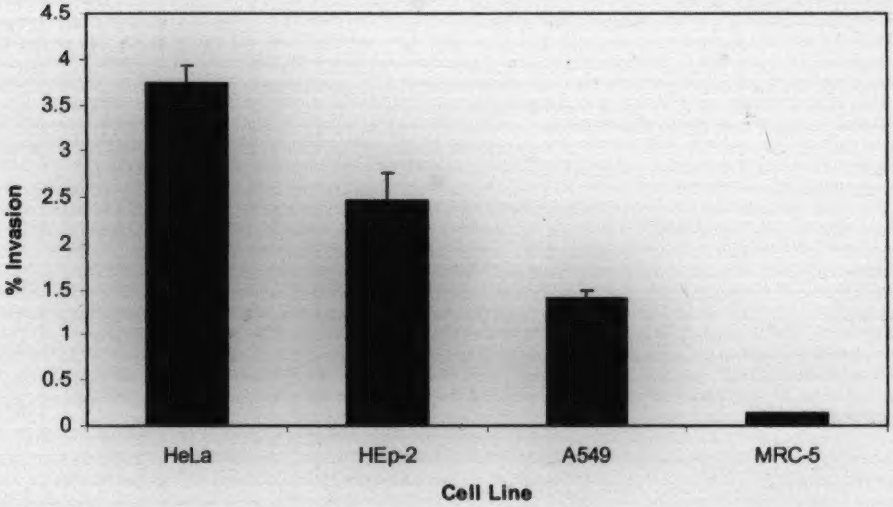

Fig. 2.1. Invasion of GBS NCS13 into cultured human cell lines. HeLa, HEp-2, A549, and MRC-5 cells were grown to confluence in 24 well culture dishes and then challenged with an inoculum of 1.5 X 10^5 CFU of NCS13, and an antibiotic protection invasion assay was performed. Graphical depiction illustrates one representative experiment in which each assay condition was performed in triplicate. Error bars represent standard error of the mean (SEM).

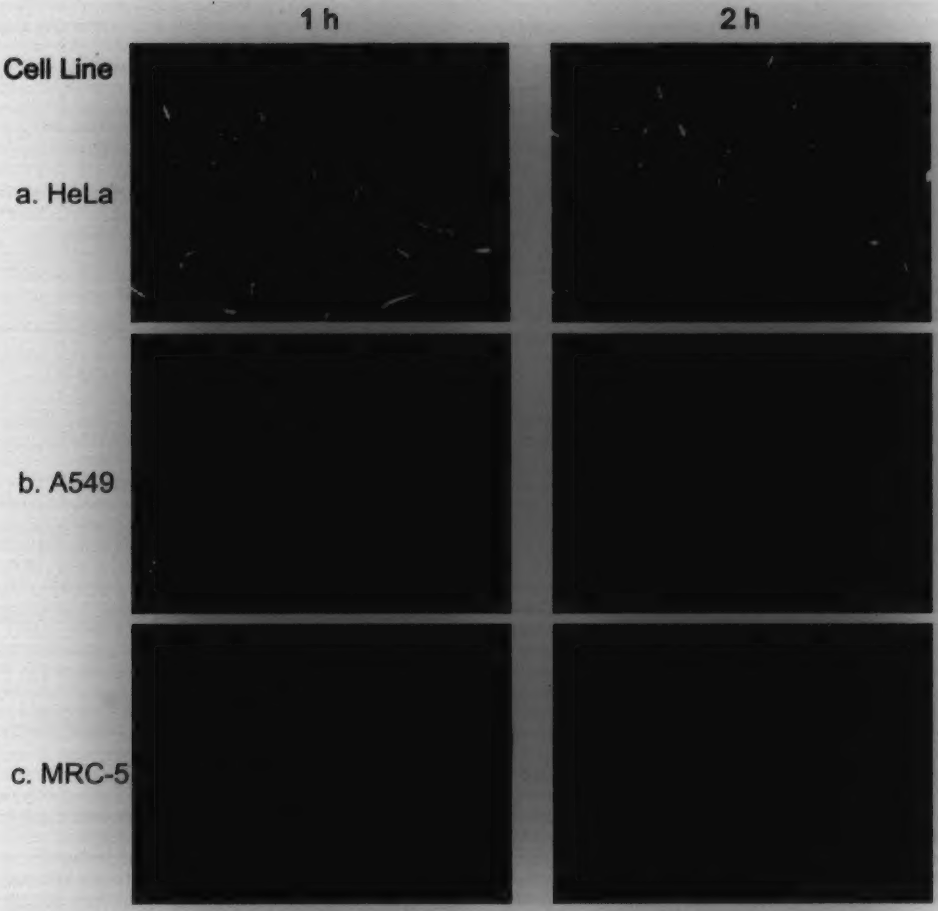

Fig. 2.2. Phase contrast microscopy of GBS NCS13 attached to cultured human cell lines. HeLa, A549, and MRC-5 cells were grown on glass coverslips in shell vials and challenged with an inoculum of 7.5 X 10^4 CFU for either 1 h or 2 h. The cells were then washed to remove unbound bacteria, fixed in a 3% formaldehyde solution, mounted on to glass slides, and visualized using phase contrast microscopy. For each assay condition, a minimum of 10 microscopic fields were examined and a representative image was taken. Bound chains are highlighted by arrows.

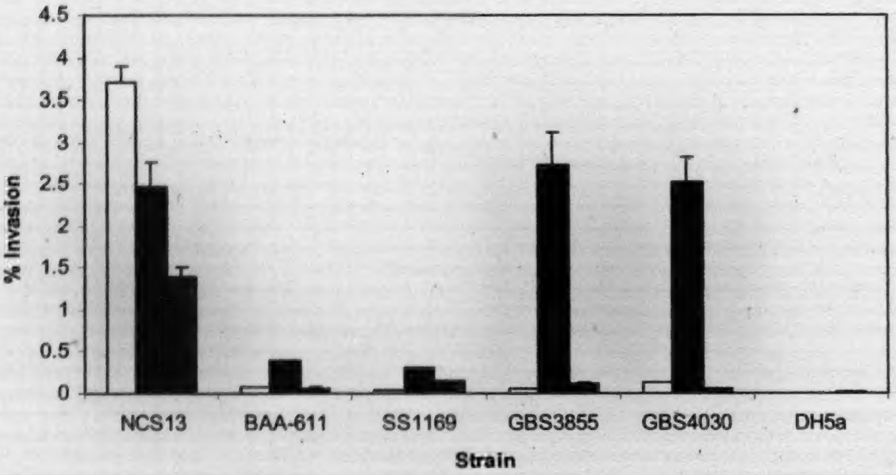

Fig. 2.3. Invasion of GBS strains into different cell lines. HeLa, HEp-2, and A549 cells were grown to confluence in 24 well culture dishes and then challenged with an inoculum of 1.5 X 10^5 CFU of 5 different GBS strains or *E. coli* DH5α and an antibiotic protection invasion assay was performed. Open bars (□), HeLa cells; closed bars (■), HEp-2 cells; Shaded bars (▪), A549 cells. Graphical depiction illustrates one representative experiment in which each assay condition was performed in triplicate. Error bars represent standard error of the mean (SEM).

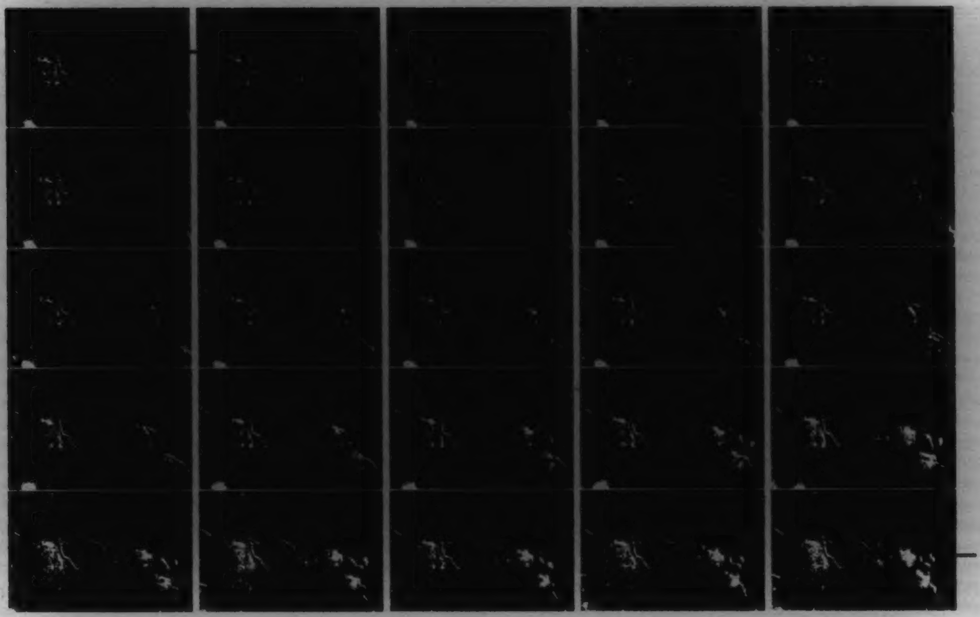

Fig. 2.4. Prolonged exposure of HeLa cells to high-intensity laser light can trigger apoptotic events. HeLa cells grown to confluence in a 35 mm MatTek culture dish were transfected with a plasmid expressing GFP-labeled actin, and infected with 3.0 X 10^7 CFU NCS13. Images of the cells were captured every 15 s using Laser Scanning Confocal Microscopy equipped with a stage warmer set to 37 °C. This figure depicts a subset of the acquired images; the first in the series is approximately one hour post NCS13 infection, with each subsequent image taken at one minute intervals. The HeLa cell in far right of each image (arrow) gradually shrinks and compacts, and then begins to form blebs consistent with an apoptotic event.

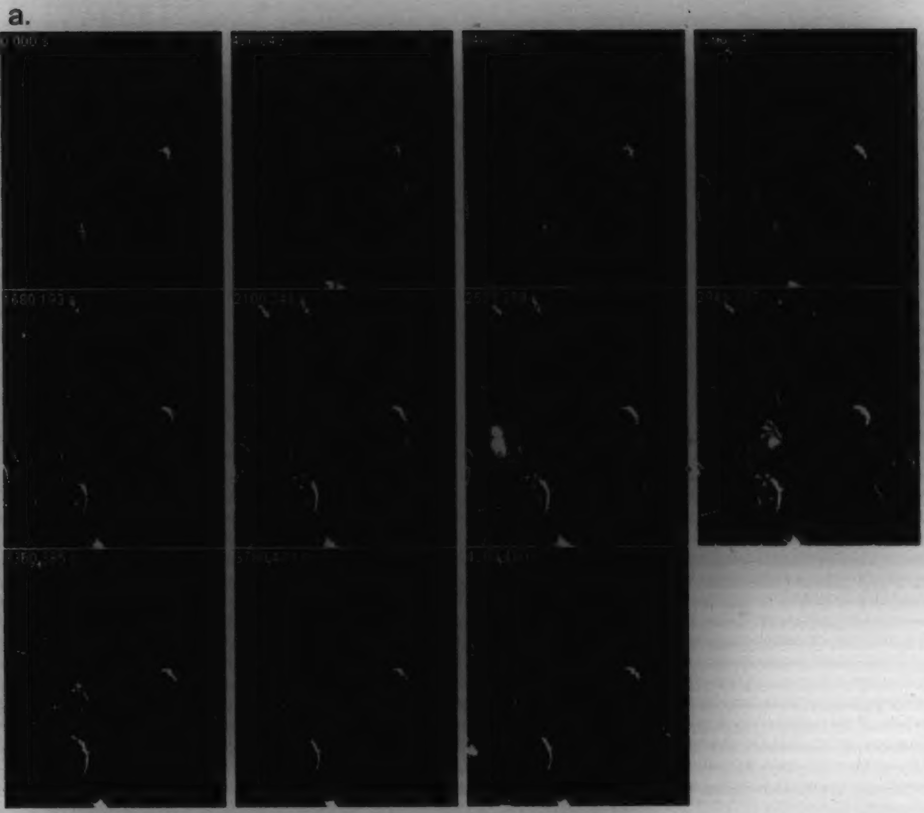

Fig. 2.5. Live microscopic analysis of GBS infecting HeLa cells. A MatTek Glass Bottom Culture dish containing HeLa cells transfected with pEGFP-actin was placed in a microscope stage warmer set to 37 °C. 2 μg of Hoescht dye (Invitrogen) was added to the dish, and then the cells were challenged with an inoculum of 3.0 X 10^7 CFU NCS13. For (a) and (b), images were captured every 60 s after infection; a subset of the images is shown. (b) is a close-up image of the HeLa cell at the bottom of the panel in (a) (arrow). Chains of NCS13 begin to attach to the HeLa cells by 840 s (14 min) post infection and by 4200 s (70 min), the final time point in the panel, most of the HeLa cells are infected by multiple NCS13 chains. In addition to the fluorescence signal from the GPF-actin and Hoescht, the digital interference contrast (DIC) image is also included in the overlay in (a) and (b).

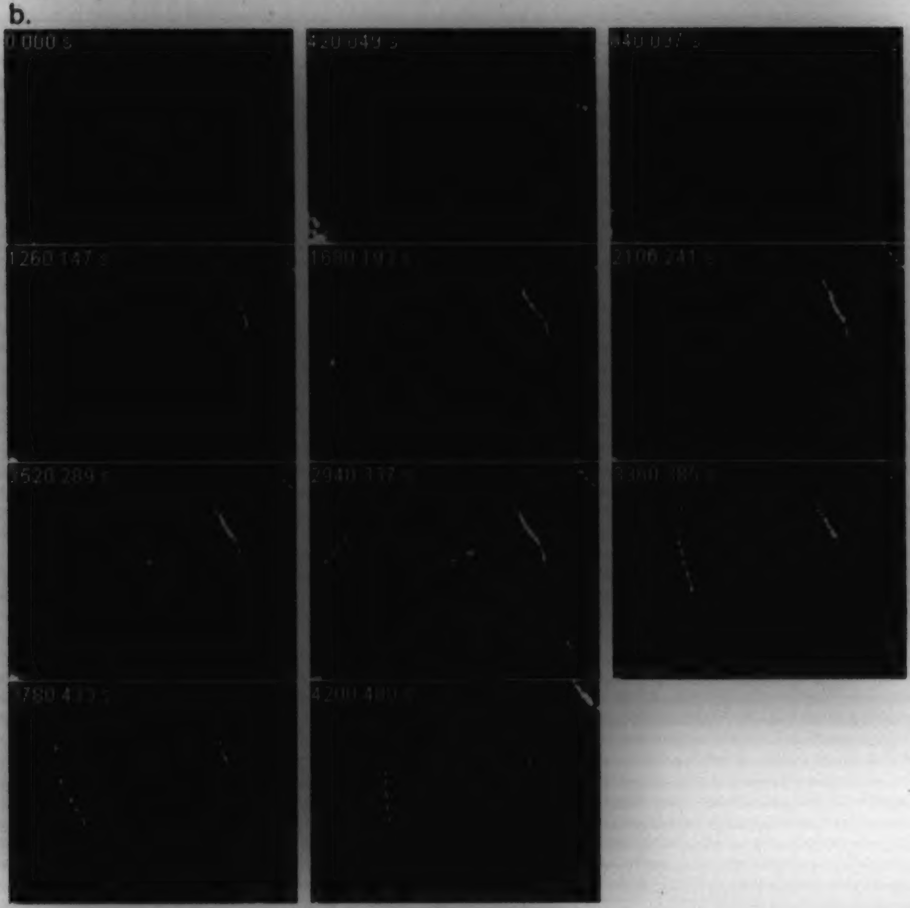
b.
0.000 s
420.049 s
840.037 s
1260.147 s
1680.193 s
2100.241 s
2520.289 s
2940.337 s
3360.385 s
3780.433 s
4200.480 s

Fig. 2.5 (continued). Live microscopic analysis of GBS infecting HeLa cells. A MatTek Glass Bottom Culture dish containing HeLa cells transfected with pEGFP-actin was placed in a microscope stage warmer set to 37 °C. 2 μg of Hoescht dye (Invitrogen) was added to the dish, and then the cells were challenged with an inoculum of 3.0 X 10^7 CFU NCS13. For (a) and (b), images were captured every 60 s after infection; a subset of the images is shown. (b) is a close-up image of the HeLa cell at the bottom of the panel in (a) (arrow in (a)). Chains of NCS13 begin to attach to the HeLa cells by 840 s (14 min) post infection and by 4200 s (70 min), the final time point in the panel, most of the HeLa cells are infected by multiple NCS13 chains. In addition to the fluorescence signal from the GPF-actin and Hoescht, the digital interference contrast (DIC) image is also included in the overlay in (a) and (b).

c.

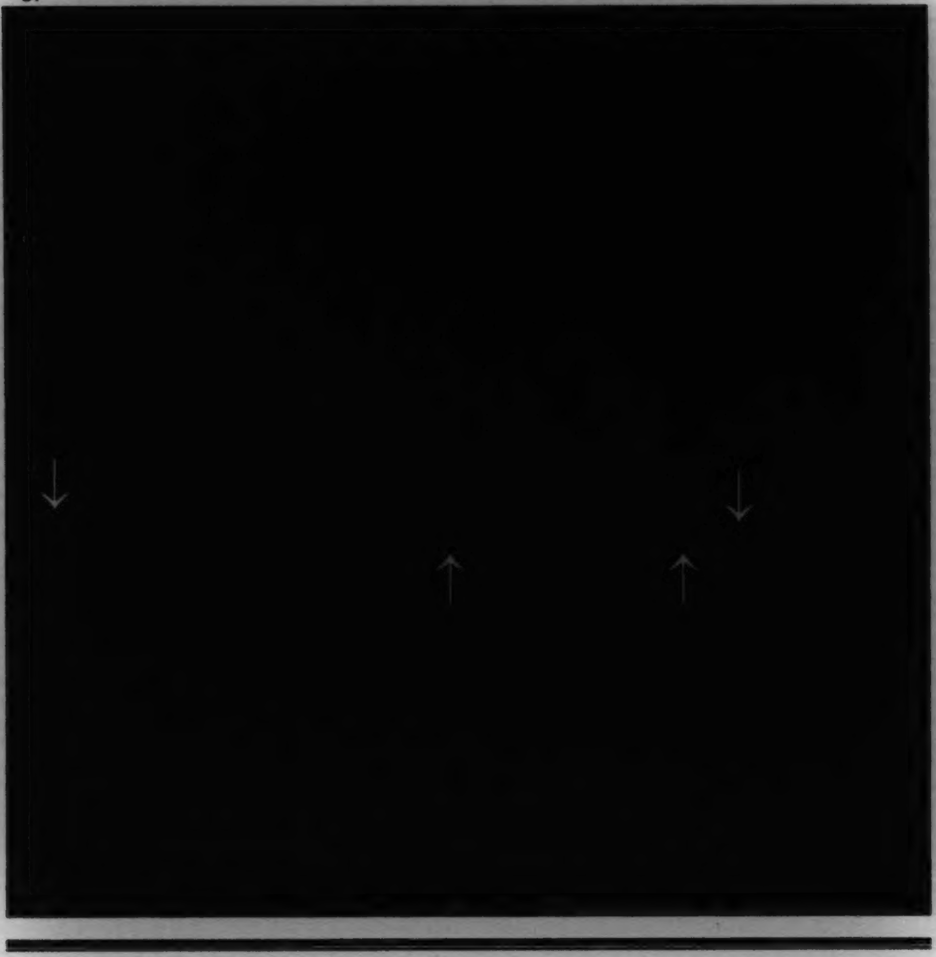

Fig. 2.5 (continued). Live microscopic analysis of GBS infecting HeLa cells. A MatTek Glass Bottom Culture dish containing HeLa cells transfected with pEGFP-actin was placed in a microscope stage warmer set to 37 °C. 2 μg of Hoescht dye (Invitrogen) was added to the dish, and then the cells were challenged with an inoculum of 3.0 X 10^7 CFU NCS13. (c). Slice images through the Z axis of NCS13 invading live HeLa Cells (arrows). An image 0.11 μM thick was captured every 0.79 μM through the Z axis. A gallery of individual slice sections is shown.

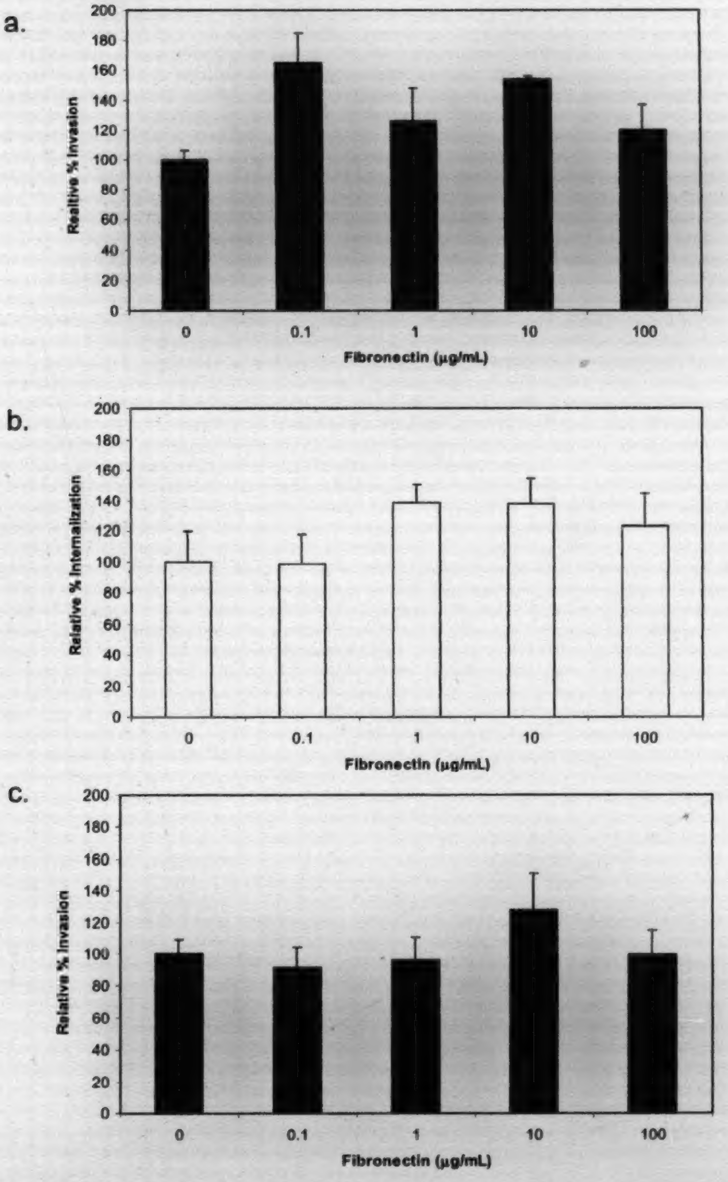

Fig. 2.6. Invasion of NCS13 pre-incubated with soluble fibronectin into host cells. NCS13 were incubated with 0, 0.1, 1, 10, or 100 µg/mL of soluble fibronectin for 30 min at 35 °C prior to performing an antibiotic protection invasion assay. (a). NCS13 invasion into HeLa cells. (b). NCS13 invasion into A549 cells. (c). NCS13 invasion into MRC-5 cells. Graphical depiction illustrates the average of two experiments in which each assay condition was performed in duplicate. The relative percent invasion was calculated as follows: [(Number of fibronectin treated GBS CFU invaded/Number of untreated GBS CFU invaded) X 100%]. Error bars represent standard error of the mean.

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Chapter 3:

Phosphoglycerate Kinase in Group B Streptococcal-Host Cell Interactions

A version of this chapter has been published as:

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3.1. Introduction

Group B streptococci (GBS) are the leading cause of meningitis in infants less than one month of age (Schuchat *et al.*, 1997). GBS are also associated with post-partum infections in the mother that can lead to bacteremia and endocarditis (Farley *et al.*, 1993;Schuchat *et al.*, 1994;Schuchat, 1998). In addition to neonatal/maternal infections, GBS are also a major pathogen in non-pregnant adults, resulting in a broad range of disease manifestations from mild skin and soft tissue infections to meningitis, pneumonia, and septicemia (Farley *et al.*, 1993;Tyrrell *et al.*, 2000).

Attachment and entry of streptococci into host cells is thought to involve a number of pathogen-host cell interactions. Streptococcal surface-associated proteins are critically important in the host-pathogen relationship as these structures can provide first contact for the bacteria with its intended host. Streptococci have been demonstrated to adhere to a variety of eukaryotic cellular structures, including laminin (Spellerberg *et al.*, 1999), fibronectin (Tamura and Rubens, 1995), fibrinogen (Beckmann *et al.*, 2002), cytokeratin (Tamura and Nittayajarn, 2000), and plasminogen (Pancholi and Fischetti, 1998). This adherence is thought to be mediated by a number of different bacterial moieties such as laminin binding protein (Spellerberg *et al.*, 1999), C5a peptidase (Cheng *et al.*, 2002;Tamura and Rubens, 1995), glyceraldehyde-3-phosphate dehydrogenase (Bergmann *et al.*, 2004;Seifert *et al.*, 2003;Winram and Lottenberg, 1996), α-enolase (Pancholi and Fischetti, 1998), and pili (Lauer *et al.*, 2005;Maisey *et al.*, 2007). Upon bacterial binding to host cell receptors, recruitment of host-cell actin to the site of bacterial entry has been observed (Ozeri *et al.*, 2001;Tyrrell *et al.*, 2002).

Actin is one of the most abundant proteins in eukaryotic cells, and is the primary determinant of cell shape and structure (Steinmetz *et al.*, 1997). It exists as a globular monomer known as G-actin and as a filamentous polymer referred to as F-actin (Steinmetz *et al.*, 1997). Alterations or modifications in cellular shape, such as pseudopod formation, are accompanied by rearrangement of actin filaments in a particular part of the cell. Actin manipulation by bacterial pathogens is a common form of pathogenic trickery, whereby the organism deceives normal eukaryotic host cell processes to effect its own entry (Ahmadian *et al.*, 2002;Cossart *et al.*, 2003;Hayward and Koronakis, 2002;Martinez *et al.*, 2000;Ozeri *et al.*, 2001;Patel and Galan, 2005;Tyrrell *et al.*, 2002). In the process of actin manipulation, other host cell components are often recruited to the site of the organism's entry.

It has been well established that GBS are able to invade a variety of host cell types, including epithelial and endothelial cells (Gibson *et al.*, 1993;Lalonde *et al.*, 2000;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997). It has also been demonstrated that invasion by streptococci is markedly reduced by host-cell treatment with cytochalasin D, a potent inhibitor of actin polymerization (Calvinho and Oliver, 1998;Dombek *et al.*, 1999;Gibson *et al.*, 1993;Greco *et al.*, 1995;Ozeri *et al.*, 2001;Talbot *et al.*, 1996;Tyrrell *et al.*, 2002). Actin recruitment to the site of bacterial attachment has been previously described in GBS and *Streptococcus pyogenes* (Ozeri *et al.*, 2001;Tyrrell *et al.*, 2002). Ozeri and colleagues demonstrated the recruitment of other host cell cytoplasmic components in addition to actin during *S. pyogenes* internalization; I hypothesized that this occurs with GBS also. Therefore I wanted to determine what other host cell components are recruited to the site of GBS bacterial entry, and the interaction between these components.

α-actinin is an actin cross-linking protein that participates in spatial organization of actin microfilaments and in the assembly of stress fibers and pseudopods (Dixson *et al.*, 2003;McGough, 1998). Initially, the objective of this chapter was to determine if α-actinin, an actin binding protein closely associated with cytoplasmic F-actin in the eukaryotic cell, plays a role in actin recruitment upon GBS internalization, and to characterize the interaction between streptococci, actin and α-actinin. However, I found that rather than the bacteria recruiting α-actinin, the α-actinin antibodies bound to the GBS surface. One of the GBS proteins to which the antibody bound is a member of the glycolytic enzyme family, streptococcal phosphoglycerate kinase (PGK). Thus, the objective of this chapter shifted to exploration of the role of PGK in GBS interactions with host cells.

3.2. Materials and Methods

3.2.1. Bacterial strains, cell lines, and growth conditions.

The GBS strain NCS13 (serotype V) was isolated from a soft tissue wound of an elderly patient and has been previously described (Tyrrell *et al.*, 2002). The *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) control strain NCTC 10241 was obtained from the National Culture Collection, PHLS Colindale, London, England. *Bacillus cereus* ATCC 14579 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Bacterial isolates were cultured on sheep blood agar plates (BAPs) (Dalynn Biologicals) or in Todd-Hewitt broth (THB) (Difco Laboratories) at 35 °C.

The human epithelial cell line HeLa 229 (ATCC CCL-2.1) was grown in OPTI-MEM I reduced serum medium supplemented with 4% fetal bovine serum (FBS) (Gibco BRL). The human epithelial cell line A549 (ATCC CCL-185) was grown in Minimal Essential Medium (MEM) supplemented with 10% FBS and 1% glutamine (Gibco BRL). Both cell lines were obtained from the ATCC.

3.2.2. Antibodies.

The number in parenthesis behind each antibody preparation indicates the dilution at which it was used. Rabbit polyclonal anti-chicken α-actinin (1:400), mouse anti-vinculin (clone VIN-11-5) (1:100), and anti-rabbit FITC conjugate (1:50) were purchased from Sigma-Aldrich. Mouse monoclonal anti-human β1 integrin (1:200), mouse monoclonal anti-human α-actinin (1:400), and anti-rabbit rhodamine conjugate (1:200) were purchased from Chemicon International. Rabbit anti-BSA antibody (1:500) was purchased from Calbiochem. Rabbit polyclonal anti-GBS antiserum (1:1000) was from Difco Laboratories; Alexa Fluor 488 anti-mouse IgG (1:100) and Alexa Fluor 568 phalloidin (1:40) were purchased from Molecular Probes.

3.2.3. Immunofluroescence microscopy of bacteria.

GBS were grown overnight in THB with shaking at 35 °C. Twenty μL of the bacterial suspension was applied to glass slides and allowed to air dry. Slides were fixed for 30 min in 3% formaldehyde, and washed in phosphate buffered saline (PBS). The slides were stained with primary antibody for 30 min at room temperature; the slides were then washed three times with PBS. Secondary antibody was added for 30 min at room temperature followed by three washes with PBS. Polyclonal anti-GBS antiserum (Difco Laboratories) was then added, followed by a secondary rhodamine-conjugated anti-IgG antibody (Chemicon International) and washed again three times in PBS. The slides were visualized using a fluorescence microscope (Olympus Microscopes, Carsen Group Inc.).

3.2.4. Actin agglutination assay.

To polymerize actin to its filamentous (F) form, 50 mM KCl (VWR International, Ltd.), 2 mM $MgCl_2$ (Fisher Ltd.) and 0.2 mM ATP (Sigma-Aldrich) were added to 1 μg/mL G-actin from bovine muscle (Sigma-Aldrich). According to the manufacturer, the actin preparation contains 90% protein (measured using the biuret method); the remaining proportion is buffer salts. Ten μL of GBS grown overnight in THB was emulsified with 10 μL of either F-actin, G-actin, PBS, or PBS with 0.2 mM ATP and examined for the presence of macroscopically visible agglutination. The preparations were then fixed using 3% formaldehyde for 30 min and examined using fluorescence microscopy.

For the fluorescence microscopy, the agglutination preparations were stained with rabbit polyclonal anti-GBS antiserum followed by goat anti-rabbit FITC conjugated secondary antibody. The slides were examined using a fluorescence microscope (Olympus Microscopes).

3.2.5. Immunoblot analysis.

Immunoblot analysis was performed as previously described (Bollag and Edelstein, 1991;Towbin *et al.*, 1992). Briefly, GBS was grown overnight in THB, washed three times in PBS and resuspended in 10 mg/mL lysozyme (Sigma-Aldrich). An aliquot was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% separating gel, and transferred to a 0.45 μM nitrocellulose membrane (Bio-Rad Laboratories Ltd.). The blots were blocked overnight in a 3% gelatin solution (Bio-Rad) and then probed with rabbit polyclonal anti-chicken α-actinin (Sigma-Aldrich) diluted 1:500 in immunoblot-blocking solution (Tris-Buffered Saline (TBS) containing 0.5% gelatin) for 2 h at room temperature. The blot was washed in TBS and then incubated in 1:10000 goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) for 1 h at room temperature. The blot washed again in TBS and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) commercial liquid substrate system (Sigma-Aldrich).

3.2.6. Peptide sequencing.

For peptide sequencing, GBS was cultured, lysed and the resulting proteins separated by SDS-PAGE as described above for the immunoblot analysis. The gel was stained with Coomassie Blue, and the 42 kDa and 55 kDa bands that corresponded to the bands on the immunoblot were excised from the gel. An in-gel tryptic digestion was carried out, and the tryptic peptides were analyzed using tandem mass spectroscopy (MS/MS). The peptide sequencing was done by the Institute for Biomolecular Design, Department of Biochemistry, University of Alberta, Edmonton, Alberta. The results obtained were analyzed using the Matrix Science Mascot program.

3.2.7. Bacterial attachment assay.

The effect of PGK on GBS adhesion to epithelial cells was assayed. Confluent monolayers of HeLa cells grown in 24 well plates were incubated with various concentrations of PGK from *S. cerevisiae* (Sigma-Aldrich) for 1 h at 35 °C. Each assay condition was performed in triplicate. GBS and *S. typhimurium* were grown in THB overnight. A 0.5 McFarland standard of each organism was made and then diluted 1 in 100 in THB. For GBS, 25 μL of this suspension (approx. 4 X 10^4 bacteria) and for *S. typhimurium* 10 μL of this suspension (approx. 1.5 X 10^4 bacteria) was added to the monolayer. The plates were centrifuged for 5 min at 100 *g* and then incubated at 4 °C for 2 h. This temperature allows bacteria to attach but inhibits epithelial cell processes required for bacterial internalization (Tamura *et al.*, 1994). Following the incubation, the cells were washed 6 times with PBS to remove any bacteria that had not adhered to the monolayer. The monolayers were trypsinized, and then lysed using 0.1% Triton X-100. The lysates were plated out onto BAPs and incubated overnight at 35 °C. The number of colonies on each plate were counted to determine the number of CFU that had attached to the monolayer. Relative percent attachment was calculated as follows: [(Number of CFU attached to PGK-treated cells/Number of CFU attached to untreated cells) X 100%].

To visualize the effect of PGK treatment on GBS attachment, HeLa cells were grown to confluence on circular glass cover-slips in shell vials. Following the 2 h incubation to allow for bacterial attachment, the cells were washed and then fixed for 30 min in 3% formaldehyde. To visualize bound bacteria, the preparations were stained with anti-GBS antisera followed by anti-rabbit FITC. To visualize the actin cytoskeleton and thus the epithelial cells, Alexa Fluor 568 labeled phalloidin was used. The coverslips were mounted onto slides, and the slides were examined visually using a fluorescence microscope (Olympus Microscopes).

3.2.8. Bacterial invasion assay.

To determine the effect of PGK treatment on the invasion of GBS into tissue culture cells, a standard antibiotic protection assay was performed with minor modifications (Rubens *et al.*, 1992;Tyrrell *et al.*, 2002). Tissue culture monolayers were grown to confluence in 24 well plates, and then treated with various concentrations of PGK from *Saccharomyces cerevisiae* (scPGK) (Sigma-Aldrich) for 1 h at 35 °C. Cells treated with bovine serum albumin (BSA) (Sigma-Aldrich) were also included as a control. Each assay condition was performed in triplicate. GBS and *S. typhimurium* were grown in THB overnight. A 0.5 McFarland standard of each organism was made and then diluted 1 in 100 in THB. For GBS, 100 μL of this suspension (approx. 1.5×10^5 bacteria) and for *S. typhimurium* 25 μL (approx. 4×10^4 bacteria) were added to the monolayer and the plates were centrifuged at 100 *g* for 5 min. The difference in inoculum is due to the fact that *Salmonellae* invade epithelial cells with higher efficiency than GBS. After a 2 h incubation at 35 °C to allow internalization, the monolayer was washed three times with PBS to remove unbound bacteria. Any bacteria that had bound but not internalized were killed by a 2 h incubation with fresh media containing 5 μg of penicillin per mL and 100 μg of gentamicin per mL. After the 2 h incubation, a 10 μL aliquot of the media was plated on a BAP to ensure that all extracellular bacteria had been killed. The monolayers were washed again with PBS, typsinized and then lysed with 0.1% Triton X-100. The lysates were plated onto BAPs and incubated overnight at 35 °C. The number of colonies on each plate was counted to determine the number of CFU that had invaded the tissue culture monolayer. Relative percent internalization was calculated as follows: [(Number of CFU invaded into PGK or BSA treated cells/Number of CFU invaded into untreated control cells) x 100%].

3.2.9. Effect of PGK treatment on epithelial cells.

HeLa cells were grown to confluence in shell vials, and then treated with either BSA, scPGK, or left untreated for 1 h at 37 °C. The cells were then washed with PBS, fixed for 30 min in 3% formaldehyde, permeabilized for 5 min with 0.1% Triton X-100, and washed again with PBS. The cells were stained with anti-vinculin for 1 h followed by anti-mouse Alexa Fluor 488 for 1 hour. This was followed by staining for 30 min with Alexa Fluor 568 labeled phalloidin. The preparations were washed, mounted onto slides and visualized using a fluorescence microscope (Olympus Microscopes).

3.2.10. Transient transfection of HeLa cells.

Transfection of HeLa cells with the vector pEGFP-actin (Clontech) was performed using Lipofectamine 2000^{TM} Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, HeLa cells were grown in 3 cm diameter Glass Bottom Culture Dishes (MatTek Corporation). When the cell density reached approximately 80% confluence, the culture media was replaced with Opti-MEM I (no FBS), and the cells were transfected with a Lipofectamine:DNA complex, with each transfection reaction containing 1 μg of plasmid DNA and 2 μg of Lipofectamine. Following transfection, the cells were incubated for 4 hours at 37 °C, and the media replaced with fresh Opti-MEM I with 4% FBS. The cells were incubated overnight at 37 °C (approximately 18-22 h) and analyzed using confocal microscopy the following day.

3.2.11. Confocal microscopy.

For live microscopic analysis of the effect of PGK on HeLa cells, the MatTek Glass Bottom Culture dish containing HeLa cells transfected with pEGFP-actin was placed in a microscope stage warmer set to 37 °C and 20 μg/mL scPGK was added to the live HeLa cells. A Carl Zeiss Laser Scanning 510 Confocal Microscope was used to image the cells; an Argon laser with a 488 nm wavelength was used to visualize GFP-actin using a 40X objective. Images were captured every 30 sec using the software program Metamorph (Universal Imaging Corp.) and further analyzed using the Zeiss LSM 5 image browser.

3.3 Results

3.3.1. Anti-α-actinin antibody binds to the surface of GBS.

Our laboratory and others have previously demonstrated that actin is recruited to the site of streptococcal attachment to eukaryotic cells (Ozeri *et al.*, 2001;Tyrrell *et al.*, 2002). Since actin is closely associated with α-actinin, I wanted to determine if this actin binding protein is also recruited to the site of GBS attachment. Using anti-α-actinin antibody, I found that α-actinin is not recruited to the site of GBS attachment, however the α-actinin antibody bound to the surface of the bacteria itself (Fig. 3.1). This antibody binding suggested that GBS possess an actin binding protein on its surface, perhaps analogous to α-actinin.

The interaction of GBS with α-actinin antibody was initially observed using a polyclonal antibody raised in rabbits against chicken gizzard α-actinin (Fig. 3.1a). The assay was repeated using monoclonal anti-human α-actinin to determine if the antibody binding was specific to a particular antibody preparation. The monoclonal anti-human α-actinin also bound to the GBS cell (Fig. 3.1b), suggesting this interaction is not restricted by the animal species to which the antibody is raised, nor the preparation of the antibody itself.

To further confirm the specificity of the antibody binding to the streptococcal surface, other antibodies were assayed for GBS binding as controls. Anti-BSA (Fig. 3.2b) or anti-β1 integrin (Fig. 3.2c) antibodies did not bind to the surface of GBS. In addition, no specific staining of the bacteria was seen with only anti-rabbit IgG FITC conjugate (Fig. 3.2d). *B. cereus* and *S. typhimurium* were also assayed for their ability to interact with the α-actinin antibody. The antibody did not bind to either of these organisms (Fig. 3.2e, f).

3.3.2. Agglutination of GBS with F-actin.

The α-actinin antibody binding to GBS suggested that this organism possesses a surface associated protein with an actin binding domain. Therefore, I wanted to determine if GBS bound actin. In muscle tissue, α-actinin cross-links actin filaments into a tetragonal lattice. I hypothesized that the protein on the GBS surface resembling α-actinin could replace α-actinin in the lattice, and that this would result in visible agglutination when streptococcal chains were mixed with F-actin. To induce the formation of actin filaments (F-actin), 50 mM KCl, 2 mM $MgCl_2$ and 0.2 mM ATP were added to G-actin solution. When equal volumes of GBS suspension and F-actin were combined, macroscopic agglutination was visible after approximately one minute (Fig. 3.3a). However, when GBS was mixed with PBS (not shown), PBS with 0.2 mM ATP (Fig. 3.3b) or G-actin (data not shown), no agglutination was observed. Microscopically, the F-actin/GBS agglutination mixture was composed of large bacteria aggregates and a lattice-type array (Fig. 3.3c). In contrast, the G-actin/GBS (Fig. 3.3d) or PBS/GBS (Fig. 3.3e) mixtures each showed random arrangement of the GBS chains with no large clumps of agglutinating cocci.

3.3.3. Western blot analysis and peptide sequencing.

Western blot was used to identify the protein(s) on the GBS surface to which the α-actinin antibody was binding. Whole bacterial cell lysates were separated by SDS-PAGE (Fig. 3.4), blotted onto nitrocellulose and probed with polyclonal anti-chicken α-actinin antibody. The anti-chicken α-actinin antibody preparation bound a 55 kDa protein and a 42 kDa protein (Fig. 3.4). The corresponding protein bands were excised from a Coomassie blue stained gel, and following an in-gel tryptic digestion, each sample was subjected to mass spectrographic analysis.

For the 55 kDa protein, the pepitdes identified from the tryptic digest were: TTLTAAITTVLARRLPTSVNQPK, GITINTAHVEYETEK, HLIVFMNK, VNDEVEIVGIK, QLDEGLAGDNVGVLLR and GQVLAKPGSINPHTK. A Matrix Science Mascot search and analysis identified these peptides as being part of the protein sequence of translation elongation factor Tu (EF-Tu) from GBS NEM316 (Glaser *et al.*, 2002). EF-Tu plays a role in bacterial protein synthesis and has been extensively described (Krab and Parmeggiani, 2002).

For the 42 kDa protein, the peptides identified from the tryptic digest were: ITAALPTIK, FDEALTGAK, VLPGLAATEK, SLAPVAADLAAK, LGQDVVFPGVTR and AHASNVGISANVEK (Table 3.1). A Matrix Science Mascot search and BLAST analysis identified these peptides as being part of the protein sequence of phosphoglycerate kinase (PGK) from GBS 2603V/R (Tettelin *et al.*, 2002). Primary amino acid sequence comparison of EF-Tu and PGK identified no obvious sequence homologies.

Although the mass spectrographic analysis identified two potential protein candidates for which the α-actinin antibody binds, my subsequent investigation focused on PGK only. This decision was based on previous reports identifying PGK as having actin binding properties (Arnold *et al.*, 1971;Bronstein and Knull, 1981) and localization of streptococcal PGK on the GBS cell surface (Hughes *et al.*, 2002).

3.3.4. Treatment of HeLa cells with PGK does not prevent GBS attachment.

GBS are known to bind to and invade HeLa cells (Greco *et al.*, 1995;Lalonde *et al.*, 2000;Tyrrell *et al.*, 2002). Presumably, if GBS were employing surface associated PGK as an adhesin to bind to host cell receptors, adding exogenous PGK to HeLa cells would competitively inhibit GBS attachment to this cell line. To examine this hypothesis, HeLa cells were pre-incubated with commercially prepared scPGK for 1 h prior to adding GBS to the monolayer and allowing the bacteria to bind to the cells. PGK from GBS and scPGK share 47% amino acid identity and 71% amino acid similarity. scPGK is readily available commercially as a standard solution, hence it was chosen for use in this assay. *S. typhimurium* was used as a control in this assay as bacterial-host cell interactions for this organism have been relatively well characterized, and are different than those proposed for streptococci (Criss and Casanova, 2003;Finlay and Cossart, 1997;Gruenheid and Finlay, 2003;Unsworth *et al.*, 2004).

As seen in Fig. 3.5, there was very little variance between the number of GBS or *S. typhimurium* attaching to untreated control HeLa cells, or to HeLa cells treated with 10 or 20 μg/mL of scPGK. This result was confirmed microscopically for GBS. After examining a minimum of ten microscopic fields for each assay condition, no visual difference in the amount of GBS attached could be detected with or without scPGK treatment. An example of this is shown in Fig. 3.5b, c. This strongly suggested that the PGK-actin interaction is not involved in the initial adhesion of GBS to epithelial cells.

3.3.5. scPGK inhibits GBS invasion into epithelial cells.

To evaluate a potential role for PGK in the epithelial cell invasion process, a modification of a standard antibiotic exclusion invasion assay was used. HeLa cells were either left untreated, or treated with 10, 20, 30, or 50 μg/mL of scPGK for 1 h. The cells were then infected with bacteria for 2 h, washed and the media replaced with fresh, antibiotic containing media for an additional 2 h to kill any bacteria that had not internalized. The HeLa cells were lysed, the resulting solution plated out, and the number of bacteria internalized was calculated. As a control, *S. typhimurium* invasion was assayed concurrently under identical HeLa cell treatment conditions. As an additional control, the effect of BSA (at concentrations equal to the scPGK treatments) on GBS invasion was also assayed.

The invasion of *S. typhimurium* into HeLa cells was not inhibited by the scPGK treatment at any concentration assayed (Fig. 3.6). In fact, a slight increase in *S. typhimurium* internalization (20% increase at a scPGK concentration of 30 μg/mL) was observed (Fig. 3.6a). The reason for this is not known. Conversely, the scPGK treatment inhibited GBS internalization into HeLa cells in a dose dependant manner; at 10 μg/mL, invasion was inhibited by over 70%, and at 50 μg/mL scPGK GBS invasion was completely abolished (Fig. 3.6a). The BSA treatment did not affect the number of GBS able to invade the tissue culture cell monolayer; invasion was not reduced by any more than 10%, even at 50 μg/mL, the maximum concentration assayed (Fig. 3.6a). This data suggested that the PGK-actin interaction likely plays a role in the GBS internalization process.

I wanted to determine if the inhibition of GBS invasion resulting from the scPGK treatment was specific to HeLa cells, or would also be observed using other epithelial cell types. HeLa cells are typically used as the GBS maternal colonization model, and A549 cells are used to model the neonatal respiratory tract. Hence, I repeated the invasion assay to determine what effect the scPGK treatment would have on the invasion of A549 cells by GBS. I found that scPGK treatment also markedly reduced GBS invasion in this cell line. Greater than 75% inhibition of internalization was observed at 20 μg/mL scPGK (Fig. 3.6b). This suggested that the effect of PGK on GBS invasion is not restricted to one cell line, but may apply to epithelial cells in general.

3.3.6. scPGK treatment results in actin rearrangement in HeLa cells.

During the microscopic assessment of the effect of scPGK on GBS adhesion to host cells, changes to the host cell actin cytoskeleton were observed. I decided to examine this effect in more detail.

When HeLa cells were incubated with 20 μg/mL BSA, no obvious alterations in the cell cytoskeleton were observed compared to untreated control cells using phalloidin staining (Fig. 3.7a, b). Untreated and BSA treated cells were of similar size and morphology, and stress fibers were clearly visible. However, when the cells were treated with 20 μg/mL of scPGK, there were obvious cellular changes (Fig. 3.7c). The cells become rounded and slightly shrunken. The most striking change were major alterations to the actin cytoskeleton. The stress fiber pattern was disrupted, and hair like projections or "tufts" of actin radiated from the perimeter of the cells. This analysis was repeated using live HeLa cells expressing GFP-actin to capture cytoskeletal modifications in real time. By 300 s (10 min) post-scPGK treatment, some of the cells began to shrink, and by 1200 s (20 min), actin projections could be observed radiating from a number of the cells (Fig. 3.8).

I hypothesized that the scPGK induced actin rearrangements may be focal adhesions. To test this hypothesis, the HeLa cells were stained with anti-vinculin under the same treatment conditions. Vinculin is a large protein that is localized to the cytoplasmic side of focal adhesions in eukaryotic cells (Fig. 3.9) (Critchley, 2000;Djinovic-Carugo *et al.*, 1999;Pokutta and Weis, 2002). No significant difference in the vinculin staining pattern was observed between any of the assay conditions examined (Fig. 3.7). This suggested that the rearrangements were not focal adhesions. The identity of the modified cytoskeletal structures requires more investigation. It is unclear if the actin rearrangement results from PGK binding to actin, or whether the addition of PGK results in a metabolic disruption within the cells that results in modulation of the cellular cytoskeleton.

3.4. Discussion

Previous reports have demonstrated that GBS recruits actin upon binding to host cells (Gibson *et al.*, 1993;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997). Consequently I investigated what other host cell proteins may also be recruited. The protein chosen for investigation was α-actinin, an actin binding protein closely associated with F-actin. While my experimental results did not demonstrate α-actinin recruitment to the site of streptococcal attachment, the results did demonstrate that α-actinin antibody binds to the surface of GBS, suggesting that the presence of actin binding protein(s) on the GBS surface. One candidate protein identified via mass spectroscopy was phosphoglycerate kinase.

This result was unexpected, largely because PGK is typically thought of as a glycolytic enzyme that facilitates the conversion between 1,3-bisphosphoglycerate and 3-phosphoglycerate in the presence of magnesium during glycolysis (Bernstein *et al.*, 1997). Classically, glycolytic enzymes are described as soluble or cytoplasmic proteins, not as surface associated moieties. However, work by Hughes and colleagues has identified PGK as a major outer surface protein of GBS (Hughes *et al.*, 2002). Surface associated PGK has also been identified in other pathogens, such as *Candida albicans* (Alloush *et al.*, 1997) and *Schistosoma mansoni* (Lee *et al.*, 1995a;Lee *et al.*, 1995b). Also, the actin-binding properties of PGK have been previously reported. Arnold *et al.* (Arnold *et al.*, 1971) and Bronstein & Knull (Bronstein and Knull, 1981) have observed that PGK from rabbit muscle not only possesses enzymatic activity, but also has the ability to bind actin. Together, this information suggests that actin binding by PGK has been evolutionarily conserved between eukaryotes and prokaryotes.

Additionally, other streptococcal enzymatic proteins have been described on the cell surface, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hughes *et al.*, 2002;Pancholi and Fischetti, 1992;Pancholi and Fischetti, 1993;Seifert *et al.*, 2003), α-enolase (Hughes *et al.*, 2002;Pancholi and Fischetti, 1998), ornithine carbamoyltransferase (Hughes *et al.*, 2002), purine nucleoside phosphorylase (Hughes *et al.*, 2002), glucose-6-phosphate isomerase (Hughes *et al.*, 2002), and glutamine synthetase (Hughes *et al.*, 2002;Suvorov *et al.*, 1997). Like PGK, some of these enzymes have been demonstrated to be multifunctional. For example, surface-associated streptococccal GAPDH binds fibronectin, lysozyme and myosin (Pancholi and Fischetti, 1992;Seifert *et al.*, 2003). Surface associated streptococcal enolase binds to host plasmin(ogen) (Pancholi and Fischetti, 1998). The multifunctional nature of these proteins suggests that their placement on the bacterial surface evolutionarily conserved, and may be important for host-pathogen interactions.

Examination of the GBS genomes that have been sequenced reveals that there is only one copy of PGK in the GBS genome (Glaser *et al.*, 2002;Tettelin *et al.*, 2002). It is unclear how PGK is targeted to or anchored in the bacterial cell wall because the GBS PGK protein does not possess the typical LPXTG gram-positive surface protein signal sequence motif. Interestingly, some of the other streptococcal cell-surface-associated "enzymatic" proteins, such as GAPDH and enolase, are also transported to the cell surface without recognizable signal sequences. Close examination of the genomes of GBS (Glaser *et al.*, 2002;Tettelin *et al.*, 2005), *Streptococcus mutans* (Ajdic *et al.*, 2002), *S. pyogenes* (Ferretti *et al.*, 2001), and *Streptococcus pneumoniae* (Hoskins *et al.*, 2001;Tettelin *et al.*, 2001) show a similar order of genes in close proximity to PGK for all these organisms. Perhaps one or a combination of these genes may be important for PGK transport.

α-actinin stabilizes focal adhesions, integrin aggregates located at the ends of bundles of actin filaments known as stress fibers. Ozeri and colleagues investigated which host cell proteins are recruited upon the binding of *S. pyogenes* to SV80 cells (Ozeri *et al.*, 2001). These investigators found that with *S. pyogenes*, initial attachment to fibronectin sets off the actin recruitment process, leading to bacterial internalization. Actin, tyrosine-phosphorylated proteins, focal adhesion kinase, paxillin, vinculin, and talin were all recruited to the site of bacterial entry, however, α-actinin was not (Ozeri *et al.*, 2001). In addition to being closely associated with actin, α-actinin is directly associated with the cytoplasmic tail of β1-integrin, which can co-localize with extracellular fibronectin, a host receptor for streptococci. The finding that α-actinin was not part of the *S. pyogenes*-fibronectin-β1-integrin integrin assemblage was surprising. Ozeri and colleagues suggested that α-actinin is involved in *S. pyogenes* entry, but that some of the other proteins involved are disassembled shortly after bacterial entry. Hence, recruitment of α-actinin is difficult to visually capture (Ozeri *et al.*, 2001). Another possible scenario is suggested here, in which streptococcal PGK binds to and occupies the α-actinin binding site on actin, thereby preventing α-actinin recruitment.

The presence of actin has been confirmed on the surface of a number of different cells types, most notably brain endothelial cells (Accinni *et al.*, 1983;Moroianu *et al.*, 1993;Pardridge *et al.*, 1989). Thus, a competitive binding assay was utilized to investigate the role of the streptococcal PGK-actin interaction on bacterial attachment to eukaryotic cells. The assays using HeLa cells suggested that PGK is not involved in the attachment of GBS to epithelial cells. However, in a competitive internalization assay, it was found that the addition of exogenous PGK to a tissue culture monolayer prior to the addition of the GBS inoculum completely abolished GBS internalization at a concentration of 50 μg/mL. This suggested that the PGK-actin interaction plays a role in the internalization of GBS into eukaryotic cells but not in initial host-pathogen contact.

The actin rearrangement that occurs in epithelial cells treated with PGK is very distinct. Although focal adhesions have been ruled out as the agents of the actin "tufts" or "spine-like projections", the identity and composition of these structures remains unclear. Furthermore, there are numerous actin-binding domains reported in the literature, and there is considerable sequence variation amongst them. Identification of the actin-binding domain of PGK will require further study.

In conclusion, I have demonstrated that PGK on the surface of GBS is recognized by antibodies to α-actinin and that exogenous PGK can inhibit the invasion of epithelial cells by GBS. This suggests that GBS PGK acts as an actin binding protein, and therefore, the PGK-actin interaction may be important during GBS invasion into eukaryotic epithelial cells.

Table 3.1. Peptide sequences identified from the mass spectrometric analysis of the 42 kDa protein. Peptides identified by MS are underlined in the protein sequence.

1 MAKLTVKDVD LKGKKVLVRV DFNVPLKDGV ITNDNR**ITAA LPTIK**YIIEQ
51 GGRAILFSHL GRVKEEADKE GK**SLAPVAAD LAAKLGQDVV FPGVTR**GAKL
101 EEAINALEDG QVLLVENTRF EDVDGKKESK NDEELGKYWA SLGDGIFVND
151 AFGTAHR**AHA SNVGISANVE K**AVAGFLLEN EIAYIQEAVE TPERPFVAIL
201 GGSKVSDKIG VIENLLEKAD KVLIGGGMTY TFYKAQGIEI GNSLVEEDKL
251 DVAKDLLEKS NGKLILPVDS KEANAFAGYT EVRDTEGEAV SEGFLGLDIG
301 PKSIAK**FDEA LTGAK**TVVWN GPMGVFENPD FQAGTIGVMD AIVKQLGVKS
351 IIGGGDSAAA AINLGRADKF SWISTGGGAS MELLEGK**VLP GLAALTEK**

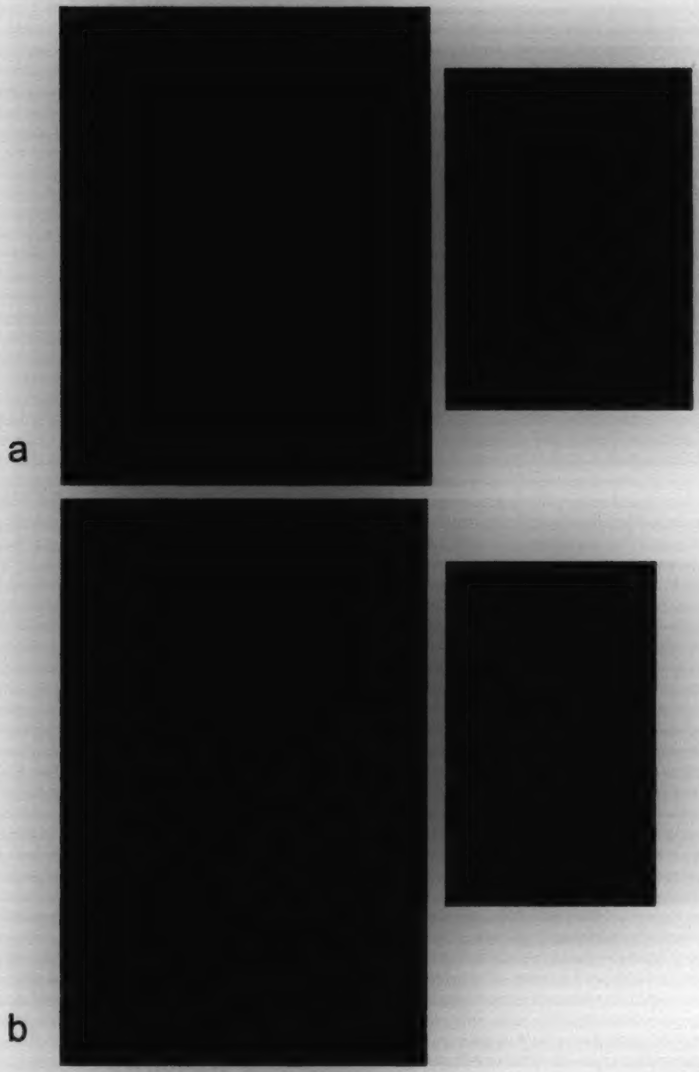

Fig. 3.1. Immunofluorescence analysis of α-actinin antibody localization on GBS. Bacteria were grown overnight in THB. A 10 μL aliquot of the bacterial suspension was placed on a glass slide. The slides were fixed in 3% formaldehyde and then stained with either rabbit anti-chicken α-actinin antibody decorated with anti-rabbit FITC conjugate or mouse anti-human α-actinin antibody decorated with anti-mouse Alexa Fluor 488 conjugate. This was followed by anti-GBS antisera decorated with rhodamine conjugate. (a). Illustrates the fluorescent imaging with anti-rabbit α-actinin (green) and (b) illustrates anti-human α-actinin localization (green) in relation to the overall streptococcal chain (red). A portion of each preparation (outlined by a blue box) was enlarged to enhance detail.

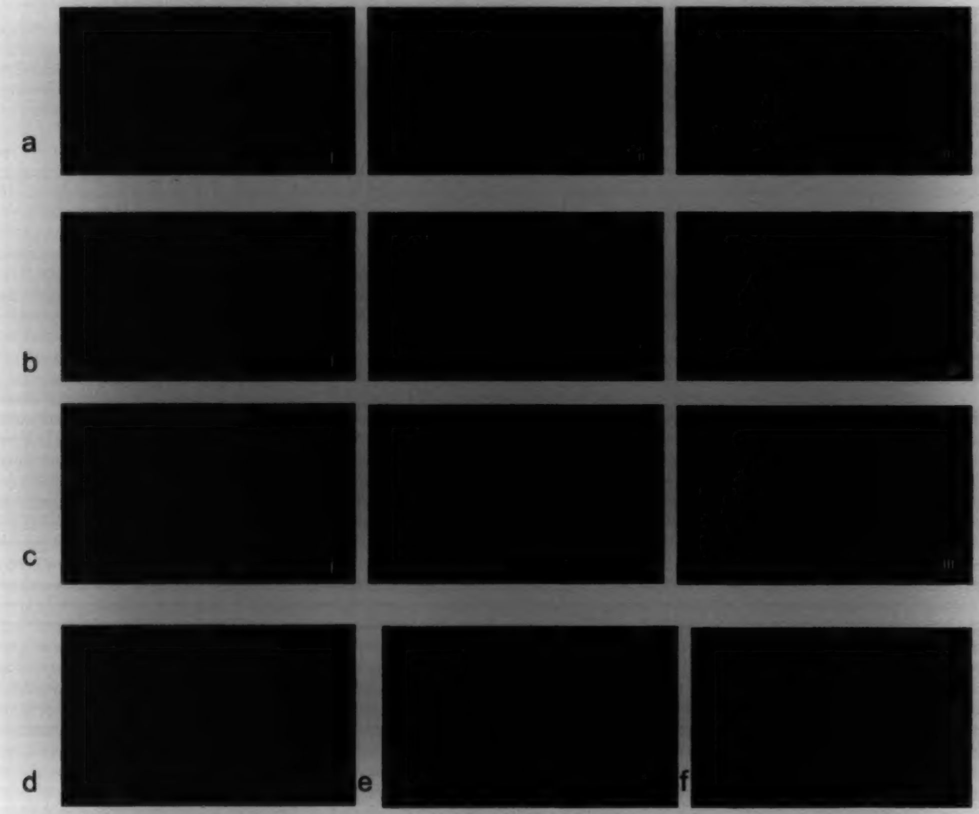

Fig. 3.2. Immunofluorescence analysis using control antibodies and bacteria. Bacteria were grown overnight in THB and 10 μL of bacterial suspensions were placed on glass slides. The slides were fixed with 3% formaldehyde prior to staining. (a). GBS were stained with rabbit anti-chicken α-actinin antibody decorated with anti-rabbit FITC conjugate (green) followed by anti-GBS antiserum decorated with anti-rabbit rhodamine conjugate (red). (ai) FITC (aii) rhodamine and (aiii) is the overlay. Bright fluorescence is seen in both color channels, illustrating that the α-actinin antibody binds to the GBS surface. (b). GBS were stained with anti-BSA antibody decorated with anti-rabbit FITC. This was followed by anti-GBS antiserum decorated with anti-rabbit rhodamine conjugate. (bi) FITC; there is no bright fluorescence of anti-BSA antibody binding to the GBS. (bii) Rhodamine; illustrating bright red fluorescence of the anti-GBS antibody binding to the streptococcal chains and (biii) is the overlay of the two channels. (Continued on next page).

Fig. 3.2 (continued). (c). GBS were stained with mouse anti-human β1-integrin decorated with anti-mouse Alexa Fluor 488 followed by anti-GBS antiserum decorated with anti-rabbit rhodamine. (ci) FITC; no bright fluorescence of the antibody binding to the GBS chains was observed. (cii) Rhodamine; bright red fluorescence of the anti-GBS antisera binding to the GBS chains and (ciii) is the overlay of the two channels. Although some green fluorescence is observed, it is diffuse background fluorescence and is not localized to the streptococcal chains. These controls illustrate that the binding of the α-actinin antibody to the GBS surface is a specific event and that non-specific antibody binding does not occur. (d). GBS stained with only anti-rabbit FITC showing that the anti-rabbit FITC conjugate does not bind to GBS, and is not responsible for the fluorescent staining seen with the α-actinin antibody. The brightness and contrast of this image have been increased to allow visualization of the preparation. *S. typhimurium* (e), *B. cereus* (f) were stained with rabbit anti-α-actinin followed by anti-rabbit FITC. The antibody did not bind to *B. cereus* or to *S. typhimurium*.

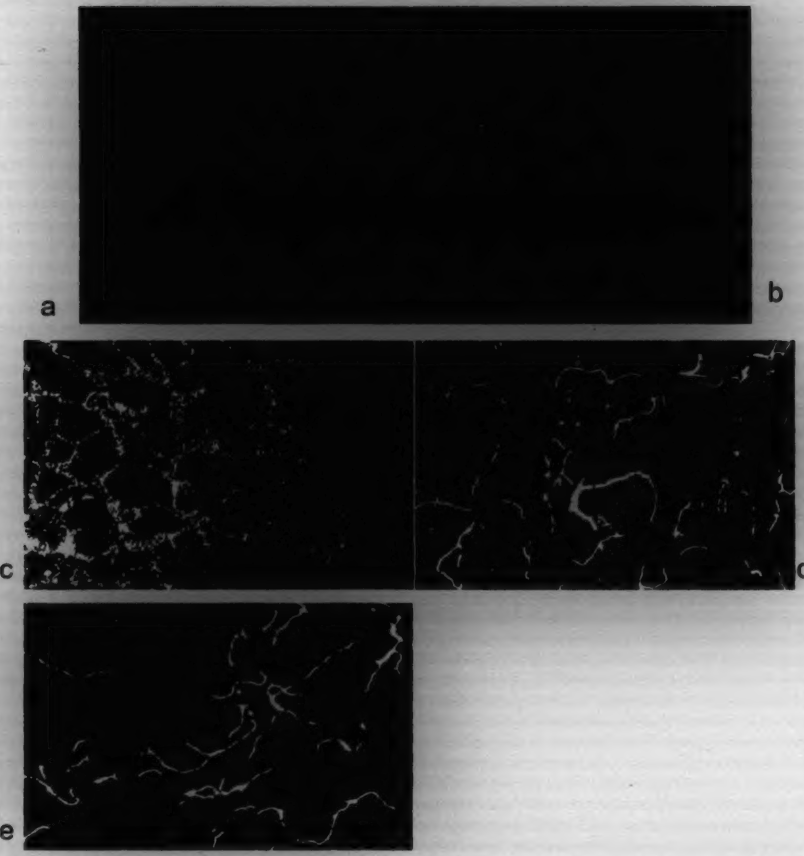

Fig. 3.3. Agglutination of GBS with F-actin. To assay the binding of streptococci to F-actin, a macroscopic agglutination assay was used. Ten μL of GBS was emulsified with an equal volume of F-actin. Visible agglutination occurred after approximately 1 min (a). This agglutination was specific to GBS mixed with F-actin. When GBS was mixed with PBS (b) or G-actin, no agglutination occurred. The agglutination assay was then fixed onto glass slides using 3% formaldehyde and stained using rabbit anti-GBS antiserum decorated with anti-rabbit rhodamine conjugate and visualized using a fluorescent microscope (black and white photo shown). As seen in (c), when GBS is mixed with F-actin, large aggregates and a lattice type of array are observed (arrows). This is contrasted with GBS mixed with PBS (d) or G-actin (e), where the GBS chains are randomly arranged, and no large clumps of agglutinating cocci are observed.

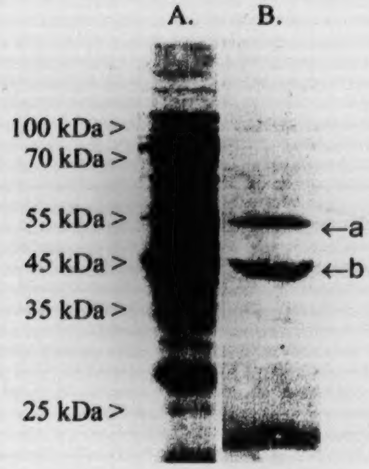
A.
B.
100 kDa >
70 kDa >
55 kDa >
45 kDa >
35 kDa >
25 kDa >
←a
←b

Fig. 3.4. Western blot analysis of GBS probed with α-actinin antibody. Whole-cell lysates of GBS were separated by SDS-PAGE (A), transferred to a nitrocellulose membrane and probed with anti-α-actinin antibody (B). The antibody bound to a 55 kDa protein (a) and a 42 kDa protein (b).

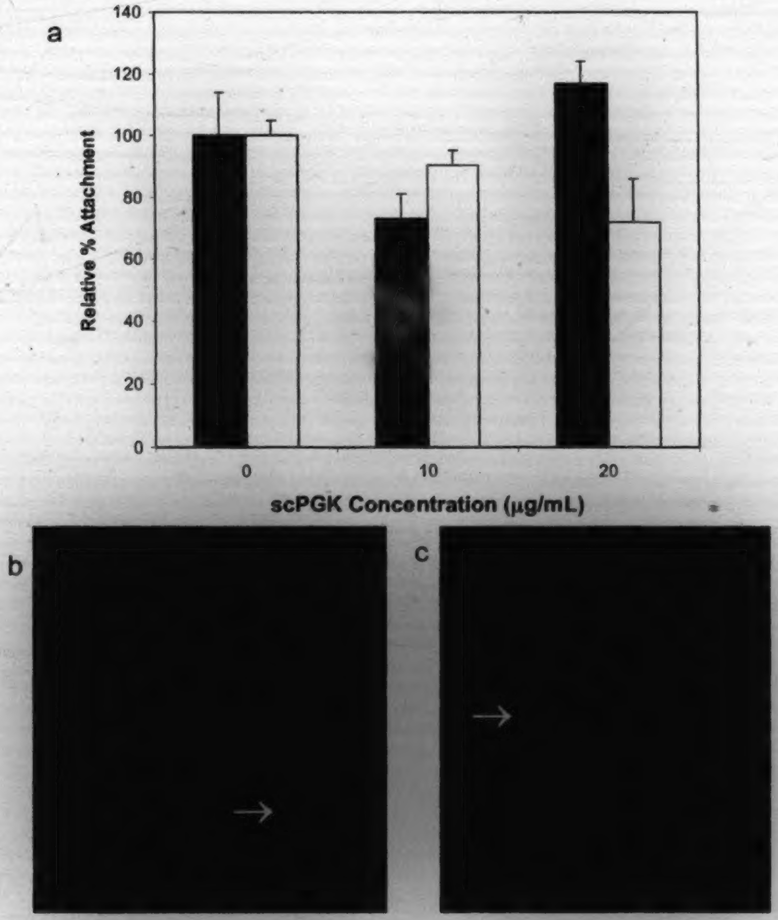

Fig. 3.5. Pre-incubation of HeLa cells with scPGK does not prevent GBS attachment. (a). Confluent monolayers of HeLa cells grown in 24 well plates were incubated with scPGK for 1 h. Following this, suspensions of GBS or *S. typhimurium* were added to the monolayer and incubated at 4 °C for 2 h to allow the bacteria to attach. The HeLa cells were then washed, lysed, and the lysates plated out onto BAPs. The number of colonies on each plate were counted to determine how many CFU were attached to the monolayer. GBS (■); *S. typhimurium* (□). Each value represents the mean of triplicate assays of one representative experiment. Vertical lines represent standard error of the mean (SEM). These results were confirmed microscopically. HeLa cells were either left untreated or treated with 20 μg/mL scPGK, infected with GBS and fixed. The bacteria were stained using anti-GBS antibody decorated by FITC conjugate. The epithelial cells were counterstained using Alexa Fluor 568 labeled phalloidin. The arrows indicate the bacteria (green) attached to HeLa cells (red). (b). Untreated control cells. (c). HeLa cells treated with 20 μg/mL scPGK.

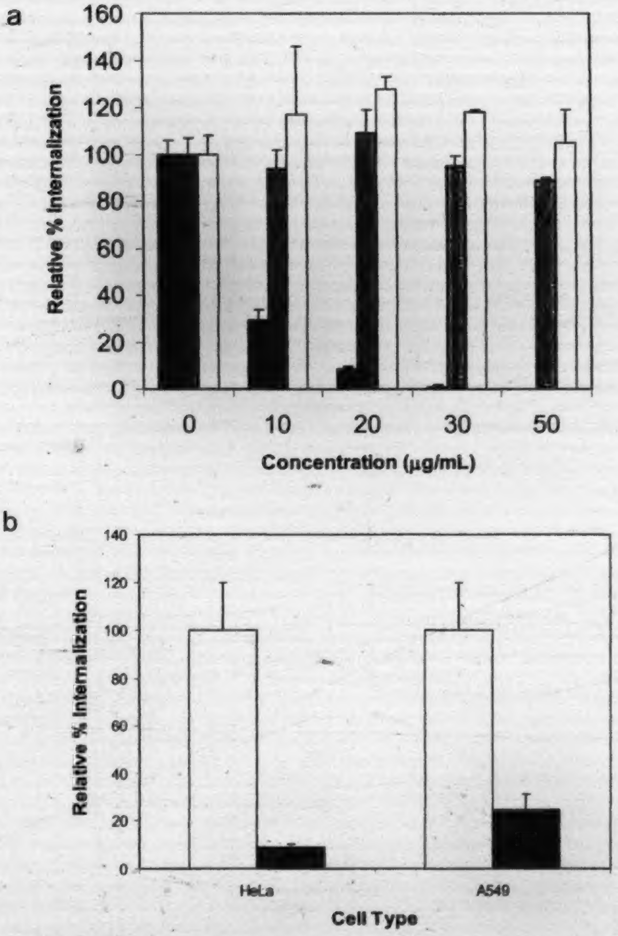

Fig. 3.6. scPGK inhibits GBS internalization into epithelial cells. HeLa cells were either left untreated, or treated with scPGK or BSA prior to infecting with GBS, or with scPGK prior to infecting with *S. typhimurium*. Following 2 h infection with the bacteria, the cells were washed and the media was replaced with fresh, antibiotic containing media to kill any bacteria that had attached but not internalized. The cells were lysed, plated to BAPs, and the number of bacteria internalized was calculated. The invasion by *S. typhimurium* (□) into HeLa cells was not diminished by the scPGK treatment. scPGK inhibited GBS (■) invasion in a dose dependant manner; invasion is inhibited by over 70% at 10 µg/mL scPGK. The BSA (■) treatment did not affect the ability of GBS to invade HeLa cells. (b) In order to determine if the scPGK inhibition of GBS invasion was specific to HeLa cells or was observed in other epithelial cell lines, the invasion assay was repeated using A549 lung epithelial cells. The A549 cells were either left untreated (□) or treated with 20 µg/mL scPGK (■). Each value represents the mean of triplicate assays of one representative experiment. Vertical lines represent SEM.

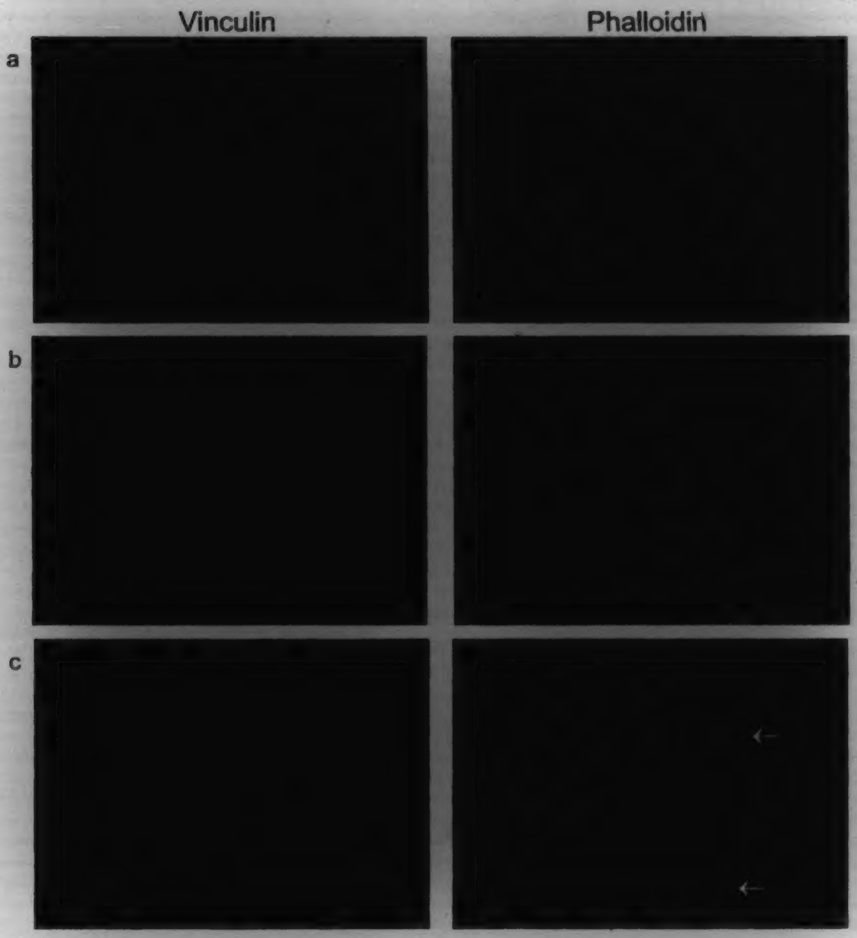

Fig. 3.7. Treating HeLa cells with scPGK results in actin rearrangement. HeLa cells were either left untreated (a), treated with 20 μg/mL BSA (b), or treated with 20 μg/mL scPGK (c). The cells were then fixed, permeabilized, and stained with anti-vinculin decorated by anti-mouse Alexa Fluor 488 followed by Alexa Fluor 568 labeled phalloidin. In the scPGK treated cells, actin rearrangement is observed, with "tufts" of actin projecting from the edges of the cells (arrows). No difference in the vinculin staining pattern is observed under any of the assay conditions.

Fig. 3.8. Live microscopic analysis of actin rearrangement in HeLa cells in response to scPGK treatment. A MatTek Glass Bottom Culture dish containing HeLa cells transfected with pEGFP-actin was placed in a microscope stage warmer set to 37 °C. 20 μg/mL scPGK was added to the cells, and images were captured every 30 sec for 1 h. A subset of the images is shown.

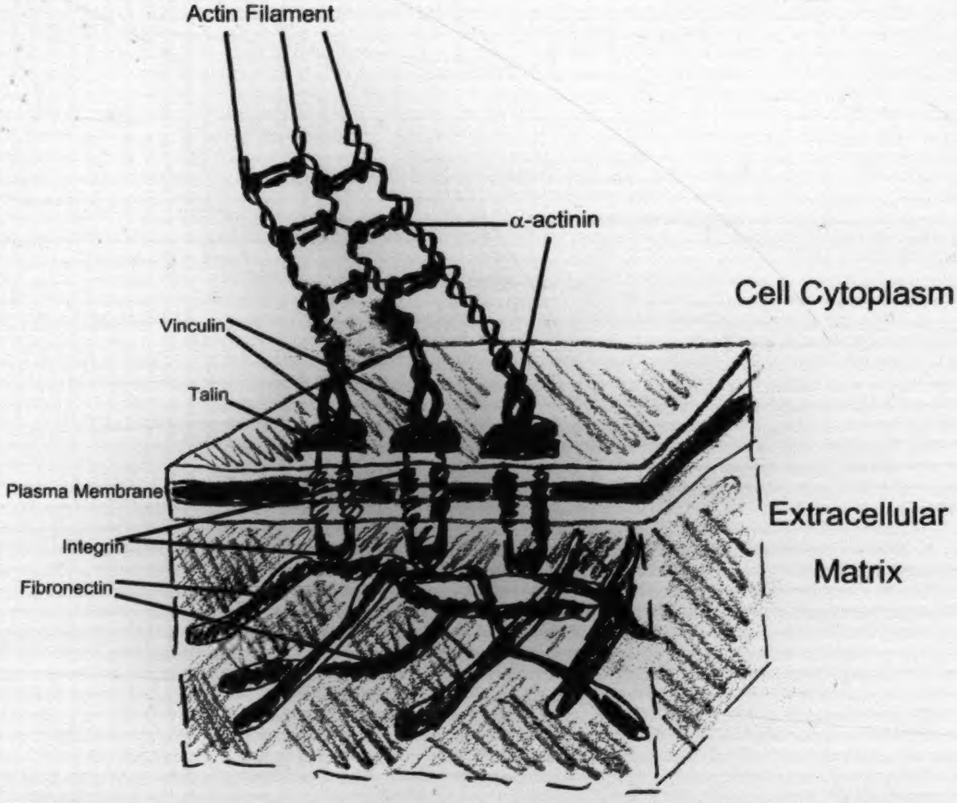
Actin Filament
α-actinin
Cell Cytoplasm
Vinculin
Talin
Plasma Membrane
Integrin
Fibronectin
Extracellular
Matrix

Fig. 3.9. A schematic representation of a focal adhesion. A focal adhesion is a macro-molecular assembly that acts as a mechanical linkage between the cellular cytoskeleton, membrane structures, and the extracellular matrix. Focal adhesions also perform as signal carriers and act as a biochemical signaling "hub" to direct numerous cellular processes based on information about the extracellular space. α-actinin cross-links actin microfilaments, and links the cytoskeleton to membrane-associated structures, including vinculin and tailin, on the cytoplasmic face. At the membrane, integrins (transmembrane proteins) indirectly interact with the cytoskeleton on the cytoplasmic side and the extracellular matrix. Adapted from Djinovic-Carugo *et al.*,1999 and the website:
http://anatomy.iupui.edu/courses/histo_D502/D502f04/lecture.f04/cell.f04/cellf04.html.

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Chapter 4:

Phosphoglycerate Kinase on the Surface of Group B *Streptococcus* Participates in Epithelial Cell Invasion and Modulates the Epithelial Cell Actin Cytoskeleton

A version of this chapter has been submitted for publication to the journal *Microbial Pathogenesis.*

4. 1. Introduction

Bacterial surface proteins can facilitate interactions with the surrounding environment, such as nutrient acquisition, binding to host immune system components, bacterial aggregation, or binding to host tissues. For an invasive bacterium, protein-protein interactions between the pathogen and host cell can be instrumental for attachment of the organism to the host cell surface, which is traditionally the precipitating event for the host cell invasion process to occur.

Enzymes that function in cellular metabolism are typically localized to the cytoplasm. These enzymes have historically been characterized as "housekeeping" enzymes, and in the past were not thought to directly contribute to the virulence of bacterial pathogens. In recent years, however, there have been a number of studies that describe glycolytic enzymes as being present on the cell surface in addition to in the cytoplasm, with evidence these proteins behave in a multifunctional manner. An example of this in bacteria is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which has been reported on the surface of Group A *Streptococcus* (GAS) (Winram and Lottenberg, 1996), *S. pneumoniae* (Bergmann *et al.*, 2004), Group B *Streptococcus* (GBS) (Hughes *et al.*, 2002;Seifert *et al.*, 2003) and *Staphylococcus aureus* (Modun and Williams, 1999). Various functions for GAPDH that contribute to pathogenesis have been characterized (Kim and Dang, 2005). Surface-associated GAPDH from GAS (termed surface dehydrogenase (SDH)) has the ability to bind human plasminogen (Lottenberg *et al.*, 1992;Pancholi and Fischetti, 1992;Winram and Lottenberg, 1996), cause ADP-ribosylation in host cells (Pancholi and Fischetti, 1993), and modulate host-cell protein phosphorylation and signal-transduction cascades (Pancholi and Fischetti, 1997).

Another glycolytic enzyme, phosphoglycerate kinase (PGK), has been demonstrated to be a surface-associated protein of the Group B *Streptococcus* (GBS) (Hughes *et al.*, 2002). Shortly after it was demonstrated that GBS PGK is found on the bacterial surface, it was observed that the addition of exogenous PGK from *Saccharomyces cerevisiae* to epithelial cells prior to GBS infection inhibited GBS invasion of these cells (Burnham *et al.*, 2005). Further work suggested that GBS PGK interacts with host-cell actin (Burnham *et al.*, 2005). It has been well documented that GBS, a Gram-positive, opportunistic pathogen, is able to invade numerous host cell types, including epithelial cells, endothelial cells, and macrophages (Gibson *et al.*, 1993;Lalonde *et al.*, 2000;Nizet *et al.*, 1997;Shin and Kim, 2006;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1996;Valentin-Weigand *et al.*, 1997). It is well established that manipulation of host cell actin microfilaments by GBS is essential for invasion to occur (Greco *et al.*, 1995;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997), however the mechanisms of this process are not completely understood.

The fact that PGK plays a central role in microbial metabolism precludes the traditional approach of creating an isogenic knock-out mutant to investigate the role of this enzyme in virulence or invasion, as such mutants are not viable. Thus an alternative approach was required to characterize the function of this "housekeeping gene" in GBS pathogenesis. The objective of this chapter was to express, purify, and characterize PGK from GBS, and to evaluate the effect of GBS-PGK on the host epithelial cell.

4.2. Materials and Methods

4.2.1. Bacterial strains, cell lines, and growth conditions.

The GBS strain NCS13 is a serotype V organism that has been previously described (Tyrrell *et al.*, 2002). The *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain NCTC 10241 was obtained from the National Culture Collection, PHLS Colindale, London, England. These organisms were cultured at 35 °C on sheep blood agar plates (BAPs) (Dalynn Biologicals) or in Todd-Hewitt broth (THB) (Difco Laboratories).

The *Escherichia coli* strains used in this study are described in Table 4.1. *E. coli* were grown on Luria-Bertani (LB) broth or agar at 35 °C (unless otherwise noted), and antibiotics (25 μg/mL kanamycin (Kan), 60 μg/mL carbenicillin (CB), or 100 μg/mL spectinomycin), were added as required.

The human epithelial cell line HeLa 229 (ATCC CCL-2.1) was grown and maintained in OPTI-MEM I Reduced Serum Medium (Invitrogen) with 4% fetal bovine serum (FBS) (Gibco), unless otherwise noted.

4.2.2. General methods.

Isolation of DNA, restriction digests, ligations, PCR, bacterial transformations, etc. were performed according to standard procedures (Sambrook and Russell, 2001) or according to manufacturer's recommendations where noted. Restriction enzymes and T4 ligase were purchased from New England Biolabs. Plasmids used in this study are listed in Table 4.2 and the sequences of the oligonucleotide primers used are in Table 4.3. All DNA sequencing was performed by the DNA Core Services Laboratory, The Department of Biochemistry, The University of Alberta using a Beckman Coulter CEQ200XL DNA Sequenator.

4.2.3. Expression and purification of PGK as a histidine-tagged fusion protein.

Expression and purification of GBS PGK was carried out according to "The QIA*expressionist*™" (Qiagen) for purification of a bacterial protein under native conditions. Briefly, genomic DNA was extracted from GBS NCS13 and the PGK gene (lacking the ATG start codon) was amplified by PCR using the oligonucleotides PGK-Bam-His and PGK-Pst-His. The 1.2 kb product was purified with the Qiaquick PCR purification kit (Qiagen) and digested with *Pst*I and *Bam*HI. The vector pQE30 was also digested with *Pst*I and *Bam*HI, both digests were purified, and PGK minus the start codon was ligated into the vector using T4 ligase. The construct was transformed into *E. coli* JM101, and transformants were recovered on LB agar plates containing CB. The resulting clones were screened using a colony blot procedure. Clones were replicate transferred to nitrocellulose that was then placed colony side up on an LB agar plate containing CB and isopropyl β-D-1-thiogalactopyranoside (IPTG); this was incubated at 35 °C for 5 h. The nitrocellulose membrane was then removed from the agar and sequentially incubated on filter papers soaked with the following solutions for the indicated times: 10% sodium dodecylsulfate (SDS) (10 Min), Denaturing Solution (0.5 M NaOH, 1.5 M NaCl) (5 min), Neutralizing Solution (1.5 M NaCl, 0.5 M Tris-Cl pH 7.4) (5 min, twice), and 2X SCC (15 min). The membrane was incubated overnight in blocking buffer (3% bovine serum albumin (BSA)) in Tris-buffered saline (TBS)). The membrane was then washed and incubated with mouse monoclonal anti-polyhistidine Clone His-1 (Sigma-Aldrich; 1:6000 dilution) for 1 h at room temperature, washed again and incubated with anti-mouse alkaline phosphatase conjugate (Sigma-Aldrich) for 1 h at room temperature, followed by washing and staining with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution for 30 min. The reaction was stopped by rinsing in dH_20. One clone exhibited a very strong positive signal; the plasmid from this clone was used in further assays and was termed pQE30-PGK.

pQE30-PGK was digested with *Bam*HI and *Pst*I to confirm the presence and correct size of the PGK insert. The plasmid-insert junctions and PGK gene were sequenced in both directions using pQE30-Seq-For and pQE30-Seq-Rev to confirm PGK was cloned in-frame with the polyhistidine tag. The pQE30-PGK construct was then transformed into *E. coli* M15[pREP4] and transformants recovered on LB agar with Kan and CB to create M15[pREP4][pQE30-PGK].

Small scale protein expression analysis was performed whereby 2 mM IPTG was added to 10 mL of log phase growth of M15[pREP4][pQE30] and M15[pREP4][pQE30-PGK] at 25 °C and 35 °C, and aliquots of the culture were taken at 0, 1, 3, and 4.5 h post-IPTG induction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the aliquots was carried out to evaluate recombinant protein expression. Following this small-scale analysis to determine an optimal time course for protein expression, larger scale PGK purification was executed. M15[pREP4][pQE30-PGK] was grown overnight in 10 mL LB with Kan and CB, and then diluted 1 in 20 with pre-warmed, fresh media to a final volume of 100 mL, and incubated at 35 °C with shaking for 1 h to achieve log phase growth. Protein expression was induced by adding IPTG to a final concentration of 2 mM for 4.5 h. The bacterial pellet was collected by centrifugation and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0). Lysozyme was added to a final concentration of 1 mg/mL and the mixture incubated on ice for 30 min, followed by sonication on ice. The lysate was cleared by centrifugation (termed cleared lysate (CL)) and then added to a 50% nickel-nitrilotriacetic acid (Ni-NTA) slurry (Qiagen), and incubated in an ice bath for 45 min with agitation. This mixture was loaded into a polypropylene column (Qiagen), and the slurry was washed with a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole (pH 8.0).

Bound protein was removed in Elution Buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole (pH 8.0). Lysis, wash and elution buffers were kept at 4 °C and 0.2% Protease Inhibitor Cocktail for His-tagged protein purification (Sigma-Aldrich) was added to each.

The concentration of purified protein was determined using the Bio-Rad Protein Assay, based on the method of Bradford (Bradford, 1976).

4.2.4. Immunoblot analysis.

For immunoblot analysis to detect polyhistidine fusion proteins, purified recombinant GBS PGK (rGBS-PGK) was resolved using SDS-PAGE with a 10% separating gel and electroblotted onto a 0.45 μM nitrocellulose membrane (Bio-Rad) following standard protocols (Bollag and Edelstein, 1991). The membrane was blocked in 5% BSA for 1 h at room temperature prior to incubation with a 1:5000 dilution of mouse monoclonal anti-polyhistidine Clone His-1 (Sigma-Aldrich) in 5% BSA overnight. For immunoblot analysis with α-actinin antibodies, whole cell lysate of GBS (grown overnight in THB) and purified rGBS-PGK were resolved using SDS-PAGE with a 10% separating gel and electroblotted onto a 0.45 μM nitrocellulose membrane (Bio-Rad) following standard protocols (Bollag and Edelstein, 1991). The membrane was blocked in 3% gelatin (Bio-Rad) for 1 h at room temperature prior to incubation with a 1:500 dilution of rabbit polyclonal anti-α-actinin antibody (Sigma-Aldrich) in 3% gelatin overnight. The following day, both membranes were washed, and incubated with anti-rabbit horseradish peroxidase conjugate for 1 h. Enhanced chemiluminescence reagent (Amersham Biosciences) was added to the membranes, and the signal was detected on BioMax Light Film for Chemiluminescence (Kodak).

4.2.5. PGK enzyme activity assay.

To determine the enzymatic activity of the 6XHis rGBS-PGK, recombinant protein was added to a reaction mixture containing 50 mM potassium phosphate, 0.83 mM glyceraldehyde-3-phosphate, 0.3 mM β-NAD, 0.2 mM ADP, 4.2 mM $MgS0_4$, 133 mM glycine, and 1 unit (U) GAPDH at 25 °C. Once the absorbance of the test solution at 340 nm (A_{340}) was constant, PGK was added and A_{340} measured at 1 s intervals for 6 min. A blank reaction where buffer was added in place of PGK was also performed. The rate of change in A_{340} in the blank was subtracted from the rate of change in A_{340} in PGK and activity in units per mL of enzyme was calculated, with one U being defined as having the ability to convert 1 μM of 1,3-bisphosphoglycerate to 3-phosphoglycerate per min at pH 6.9 at 25 °C. The activity of a commercial scPGK solution with known activity (Sigma-Aldrich) was assayed in parallel as a control.

4.2.6. Antibiotic protection (invasion) assay.

Invasion of bacteria into a HeLa cell monolayer was quantified using a standard antibiotic protection invasion assay with minor modifications (Rubens *et al.*, 1992;Tyrrell *et al.*, 2002). HeLa cells were grown to confluence in 24 well plates (Corning). The HeLa cells were then incubated for 1 h with PGK, BSA, or left untreated (as described for the various assays), with each assay condition performed in triplicate. Overnight growth of GBS or *S. typhimurium* in THB was adjusted to a 0.5 McFarland standard, and then diluted 1 in 100 in THB. From there, approximately 1.5 X 10^5 GBS or 4 X 10^4 *S. typhimurium* were added to the cell monolayer. The difference in inoculm reflects the variation in invasion efficiency of these two organisms.

Following inoculation, the cell culture plates were centrifuged at 100 *g* for 5 min at room temperature and then incubated at 35 °C for 2 h to allow bacterial internalization to occur. The monolayer was then washed three times with phosphate buffered saline (PBS) to remove unbound bacteria. Bacteria that had bound but not internalized were killed by incubation for 2 h with fresh media containing 5 µg of penicillin per mL and 100 µg of gentamicin per mL. After the 2 h incubation, an aliquot of media from each well was applied to a BAP to ensure that the antibiotic treatment was adequate to kill all extracellular organisms. The cell monolayers were then washed with PBS, trypsinized and then lysed with 0.1% Triton X-100, the lysates spread onto BAPs, and incubated overnight at 35 °C. The number of colonies on each plate was counted to assess the number of CFU that had invaded the monolayer. Portions of the HeLa cell culture supernatants were plated on to BAPs following the initial 2 h infection period to ensure that the inhibitor treatments did not reduce bacterial viability.

4.2.7. Statistical analysis.

For the invasion assays, relative percent invasion was calculated as follows: [(Number of CFU invaded into treated cells/Number of CFU invaded into control cells) X 100%]. The graphical representations are illustrative of the mean of one representative experiment in which each assay condition was performed in triplicate; each assay was performed at least 3 times. GraphPad InStat3 software was used for statistical analysis of the data; analysis of variance using Dunnet's Multiple Comparisons Test was used to compare invasion into treated cells to that into control cells. A *P* value < 0.05 was considered statistically significant. Error bars represent standard error of the mean (SEM).

4.2.8. Construction of the plasmid pFW11-PGKB-Tail.

A web-based "Transmembrane Prediction Server" (http://www.sbc.su.se/~miklos/DAS/) was utilized to design a hydrophobic peptide sequence that would have properties similar to a transmembrane protein segment based on a "Dense Alignment Score" (DAS) (Cserzo *et al.*, 1997). A DAS of 2.2 or greater is considered to be highly predictive of a transmembrane segment. Examination of surface proteins in the GBS genome revealed a "cell wall surface anchored family protein" (SAG1407) (Tettelin *et al.*, 2002) with a C-terminal sequence that possessed a good fit for this application; it was altered slightly to generate an optimal DAS (Fig. 4.1). No transmembrane-like segments are predicted to exist in the wild-type GBS PGK sequence, but the addition of this *de* *novo* hydrophobic sequence to PGK results in a DAS well within the predicted transmembrane zone (Fig. 4.1b).

A vector housing the GBS PGK containing a hydrophobic tail sequence was constructed in two steps. First, the PGK gene was amplified by PCR from NCS13 (Sal-PGK-New-R, Bam-PGK-New-F). The purified PCR product and the vector pFW11 were each digested with *Sal*I and *Bam*HI, purified and then ligated using T4 ligase. The mixture was transformed into *E. coli* DH5α, and transformants were selected for on spectinomycin agar. Clones were screened by restriction digestion analysis and resulting construct pFW11-PGKB was identified. The Stratagene QuikChangeII® Site-Directed Mutagenesis Kit was used according to the manufacturer's recommendations, and the oligonucleotides HB-Tail-F2 and HB-Tail-R2 were used to add a hydrophobic tail sequence to the C-terminal end of the PGK gene. The resulting construct was transformed into *E. coli* XL1-Blue. The addition and orientation of the hydrophobic sequence was confirmed by sequencing with the sequencing primer PGK-SEQ-R-1 and the resulting construct was designated pFW11-PGKB-Tail.

4.2.9. Cloning Green Fluorescent Protein (GFP) constructs.

The GFP Fusion Topo® TA Expression kit (Invitrogen) was used to clone PGK and variants as GFP fusion proteins for expression studies according to the manufacturer's instructions. The PGK gene lacking the start codon was amplified by PCR from NCS13 genomic DNA (GFP-PGK-F, Bam-PGK-C-R) and PGK with a hydrophobic tail sequence was amplified from pFW11-PGKB-Tail (GFP-PGK-F, PGK-Tail-Asc-R). The resulting products were purified, cloned into pcDNA3.1/NT-GFP-TOPO, transformed into *E. coli* TOP10 cells, and transformants recovered on CB agar. Orientation of the inserts was confirmed via restriction analysis, PCR, and sequencing of the plasmid with the primer GFP Forward. The resulting constructs were designated pGFP-PGK, pGFP-PGKrev and pGFP-PGKTail.

4.2.10. Transfection and visualization of HeLa cells expressing GFP-PGK and GFP-PGK variants.

Transfection of HeLa cells with the GFP-PGK constructs was performed using Lipofectamine 2000^{TM} Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, HeLa cells were grown on sterile glass coverslips in 24 well cell culture dishes. When the cells had grown to approximately 90% confluence, the culture media was replaced with Opti-MEM I (no FBS), and the cells were transfected with a Lipofectamine:DNA complex with each transfection reaction containing 1 μg of plasmid DNA and 2 μg of Lipofectamine. Following transfection, the cells were incubated for 5, 18, or 28 h, and then fixed in a 3% formaldehyde-PBS solution for 30 min at room temperature, with 2 μg of Hoescht 3342 dye (Invitrogen) added to each coverslip to label the cell nuclei. The cells were permeabilized with a 0.1% Triton X-100-PBS solution for 5 min, washed and then stained with Alexa Fluor 568 labeled phalloidin to fluorescently label filamentous actin for 20 min at room temperature. The coverslips were mounted onto glass slides, and visualized using a fluorescence microscope (Olympus Microscopes, Carsen Group Inc.).

4.3. Results

4.3.1. Expression and purification of recombinant GBS PGK (rGBS-PGK).

The GBS PGK gene lacking the start codon was amplified by PCR and cloned into the vector pQE30, creating pQE30-PGK for PGK overexpression as a N-terminal hexahistidyl (6XHis) derivative. A 6XHis expression scheme was selected as this motif is small, in most cases does not interfere with protein folding or function, and facilitates the production of a highly purified product. Small scale protein expression analysis was performed and SDS-PAGE analysis of the expression cultures revealed rGBS-PGK expression in the M15[pREP4][pQE30-PGK] lysates at 1 h following induction by IPTG, and at 4.5 h, the recombinant protein comprised a large proportion of the total cellular lysate (Fig. 4.2a). In contrast, no PGK expression was observed in M15 with pQE30 vector alone or in the uninduced culture (time 0 h) (Fig. 4.2a). Recombinant protein expression also occurred if the culture was grown at 25 °C (not shown). Based on these results, M15[pREP4][pQE30-PGK] at 4.5 h post IPTG induction, grown at 35 °C, was used for rGBS-PGK purification.

The rGBS-PGK was purified by affinity chromatography using the Qiagen system Ni-NTA column (Fig. 4.2b). The protein yield from multiple purification assays was a range from 3 to 4 mg of rGBS-PGK per 100 mL of M15[pREP4][pQE30-PGK] culture (corresponding to a M15 bacterial pellet with a wet weight of approximately 2 g).

Faint protein bands below PGK were observed in the Coomassie stained SDS-PAGE gel (Fig. 4.2b, E2-E4). An aliquot of rGBS-PGK from elution fraction E3 as well as E3 diluted 1/100 were analyzed by Western blot for reactivity with anti-polyhistidine antibody. In addition to the rGBS-PGK, the smaller protein species showed strong reactivity with the anti-polyhistidine antibody (Fig. 4.2c), indicating that these are degradation or truncation fragments of rGBS-PGK, and do not represent other protein species.

4.3.2. Purified rGBS-PGK is an enzymatically active protein.

Once purified, rGBS-PGK was assayed to determine if it was enzymatically functional. rGBS-PGK activity was determined by measuring the production of NADH at 25 °C as an increase in absorbance at 340 nm based on the following reaction:

$$\text{GAP} + \beta\text{-NAD} + P_i \xrightarrow{\text{GAPDH}} \text{1,3-Bisphosphoglycerate} + \beta\text{-NADH}$$

$$\text{1,3-Bisphosphoglycerate} + \text{ADP} \xrightarrow{\text{PGK}} \text{3-Phosphoglycerate} + \text{ATP}$$ (Bucher, 1955).

Commercially available PGK (Sigma-Aldrich) from *S. cerevisiae* (scPGK) with stated activity was assayed in parallel as a control. One U is defined as the amount of PGK required to convert 1 μM of 1,3-bisphosphoglycerate to 3-phosphoglycerate per min at pH 6.9 and at 25 °C. The activity of the purified rGBS-PGK was found to be 200 U per mg protein. The enzymatic activity of rGBS-PGK was abolished if the protein was boiled for 5 min or treated with a 1% SDS solution for 5 min prior to performing the activity assay.

4.3.3. rGBS-PGK reacts with α-actinin antibody by Western blot.

Initially, GBS PGK was identified as a potential actin-interacting moiety because of its cross-reactivity with α-actinin antibodies, both on the surface of GBS by immunofluorescence, and during Western blot analysis of crude whole-cell GBS lysates (Burnham *et al.*, 2005). Based on these observations, and to determine if the rGBS-PGK possessed these same epitopes, the ability of rGBS-PGK to cross-react with α-actinin antibodies was assayed. Crude whole-cell GBS NCS13 lysate and purified rGBS-PGK elution fraction E3 were Western blotted and probed with anti-α-actinin antibody (Fig. 4.2d). The α-actinin antibody bound a protein species of approximately 42 kDa in the GBS NCS13 lysate as previously reported (Burnham *et al.*, 2005) and the purified rGBS-PGK, suggesting that the rGBS-PGK possess the same α-actinin cross-reactive epitope found on GBS.

4.3.4. Treatment of HeLa cells with rGBS-PGK prior to GBS infection inhibits GBS invasion.

In my previous investigation, it was observed that treatment of epithelial cells with scPGK prior to infection with GBS resulted in a reduction in GBS invasion without affecting GBS attachment (Burnham *et al.*, 2005). Thus, I sought to determine if treatment of HeLa cells with rGBS-PGK would affect GBS invasion of HeLa cells in a manner similar to scPGK. The invasion of HeLa cells by *S. typhimurium* was assayed in parallel as a control, as this organism is known to invade via different mechanisms than GBS, and the invasion of *S. typhimurium* into HeLa cells is not reduced in response to scPGK treatment (Burnham *et al.*, 2005). HeLa cells were either untreated, or treated for 1 h prior to bacterial infection with BSA, scPGK, rGBS-PGK or PGK buffer (Fig. 4.3). The addition of 30 μg/mL BSA or PGK buffer alone did not reduce GBS or *S. typhimurium* invasion (Fig. 4.3). In contrast, treating the HeLa cells with PGK reduced GBS invasion but not *S. typhimurium* invasion; 30 μg/mL scPGK (corresponding to 50 U) reduced GBS invasion by approximately 90%, and 30 μg/mL rGBS-PGK (corresponding to 4 U) reduced GBS invasion by approximately 40% (Fig. 4.3). The addition of 300 μg/mL rGBS-PGK (corresponding to 40 U) reduced GBS invasion by approximately 50%; this reduction in invasion was considered significant (*P* value < 0.01 compared to untreated control cells) (Fig. 4.3).

4.3.5. Inactivation of scPGK does not impair its ability to inhibit GBS invasion of epithelial cells.

Although the level of inhibition of GBS invasion observed in response to the rGBS-PGK is significant, it is less than that imparted by the scPGK. I postulated that this may be due to differences in the enzymatic activity between the two PGK preparations. To investigate this further, scPGK was boiled for 5 min to inactivate it prior to its addition to the HeLa cell monolayer. Boiled BSA was assayed in parallel as a control. It was found that at 20 μg/mL and 40 μg/mL, the inactivated scPGK did inhibit GBS invasion, and that the level of inhibition imparted by the boiled scPGK did not differ from native scPGK (Fig. 4.4). The addition of boiled BSA to the HeLa cells prior to GBS invasion did not affect GBS invasion of this cell line. The invasion of *S. typhimurium* was not altered significantly by any of the cell treatments (Fig. 4.4).

4.3.6. Expression of GBS-PGK inside of HeLa cells disrupts the actin cytoskeleton.

My previous investigations found that when HeLa cells are incubated with scPGK, disruption of the HeLa cell actin cytoskeleton occurred (Burnham *et al.*, 2005). scPGK disrupted the stress fiber pattern, and "tufts" of actin radiated from the edges of the HeLa cells (Burnham *et al.*, 2005). In addition, the cells became rounded and compact (Burnham *et al.*, 2005). To explore the effect of GBS PGK on the HeLa cell actin cytoskeleton, the PGK gene lacking a start codon was amplified by PCR from GBS NCS13 and cloned into the eukaryotic expression vector pcDNA3.1/NT-GFP-TOPO® for expression as a Green Fluorescent Protein (GFP) fusion protein, with GFP on the N-terminus of PGK (pGFP-PGK). As a control, PGK was also cloned in the antisense direction as an N-terminal GFP fusion (pGFP-PGKrev). The constructs were transfected into HeLa cells and pEGFP-actin (Clontech) was also transfected in parallel as a control of the transfection procedure. A GFP Expression Control (expressing only GFP) was also assayed.

No GFP expression was observed at 5 h for any of the transfection reactions assayed (not shown). The transfection efficiency for pEGFP-actin was approximately 70% (Fig. 4.5a) at 18 and 28 h. In contrast, the transfection rate with pGFP-PGK was much lower (approximately 10 to 15% of the total cell population). The reason for the lower transfection efficiency observed with this construct is not clear. A possible explanation is that expression of GBS-PGK at high levels inside the HeLa cell is toxic to the cell. There was no detectable variation in the proportion of transfected cells, or the effect of expressing the fusion protein in the cells between the 18 or 28 h time points (not shown).

In the HeLa cells expressing GFP-PGK, the cytoskeleton is modified (Fig. 4.5 c and d). The stress fiber pattern is disrupted and an accumulation of filamentous actin at the peripheral edges of the HeLa cells was observed (Fig. 4.5 c and d). In addition, most of the transfected cells are compacted or ovoid in shape, in comparison to the untransfected surrounding cells. In contrast, HeLa cells expressing GFP-PGKrev (PGK cloned in the antisense direction) did not exhibit a modified cytoskeleton (Fig. 4.5 e and f), nor did the HeLa cells expressing only the GFP expression control (Fig. 4.5b), suggesting that the disruption of the cytoskeleton observed in response to pGFP-PGK is a specific consequence of the expression of GBS PGK inside of the HeLa cell.

4.3.7. The addition of a hydrophobic peptide to the C-terminus of GBS-PGK prior to expression inside the HeLa cell abolishes PGK-induced cytoskeletal disruption.

The observation that inactivation (boiling) of scPGK did not affect its ability to inhibit GBS invasion into HeLa cells suggested that modulation of GBS-epithelial cell invasion by PGK is not mediated by the enzymatic activity of PGK inside the host cell, but rather may be based on a protein-protein interaction. Thus, I sought to express a mutant form of GBS PGK and explore the effect of the mutation on the ability of GBS PGK to disrupt the host cell cytoskeleton. However, since the nature of this protein-protein interaction is unknown, devising a strategy for creation of targeted mutations in the PGK protein would be difficult.

Mutant PGKs from different species that lack enzymatic activity have been created, however the finding that inactivated (boiled) PGK retained its ability to inhibit GBS invasion suggested that mutations focused on GBS PGK enzymatic activity were unlikely to be informative in this context. An ideal mutant form of GBS PGK would be one lacking in actin binding ability (Burnham *et al.*, 2005), however protein actin-binding domains vary widely in the literature, and no such domain was obviously revealed amongst the primary amino acid sequence of PGK after querying a variety of protein domain search algorithms. Thus, I assayed the effect of modifying the GBS PGK C-terminus by the addition of a hydrophobic peptide sequence. A similar modification was used to investigate another multifunctional, anchorless streptococcal enzyme, the GAS SDH (Boel *et al.*, 2005), and it was observed that the addition of a hydrophobic tail to SDH altered the compartmentalization of SDH within GAS, and the GAS SDH mutant strain was less able to bind human plasminogen and human pharyngeal cells (Boel *et al.*, 2005).

The mutant PGK possessing a C-terminal hydrophobic sequence (Fig. 4.1) was cloned as a GFP-fusion to generate pGFP-PGKTail, and the resulting construct transfected into HeLa cells for expression. The pattern of GFP fluorescence in HeLa cells expressing pGFP-PGKTail was markedly different from those expressing pGFP-PGK. In the cells expressing the mutant construct, the GFP fluorescence was largely localized in a peri-nuclear halo and did not reach the periphery of the cell (Fig. 4.6 a and b). There also appeared to be "bubbles" or "circles" of GFP fluorescence and a pattern of GFP expression resembling a lace-like design, as opposed to the more homogenous GFP distribution observed in the wild-type PGK expressing cells (Fig. 4.6 a and b, Fig. 4.5 c and d). Furthermore, the cells expressing the mutant PGK did not exhibit disruption of the HeLa cell cytoskeleton; the stress fiber pattern was not altered and the actin cytoskeleton of the transfected cells did not appear different from the surrounding untransfected cells (Fig. 4.6 a and b).

4.4. Discussion

Phosphoglycerate kinase (PGK), a glycolytic enzyme whose protein sequence and structure are highly conserved among both prokaryotic and eukaryotic species, has been described as a major outer surface protein of Group B *Streptococcus* (GBS) (Hughes *et al.*, 2002). Previous work in our laboratory found that the addition of scPGK to HeLa cells prior to GBS infection was inhibitory to GBS invasion of these cells (Burnham *et al.*, 2005) and hence I was interested in determining the effect of GBS-PGK on GBS invasion of HeLa cells. Sequencing of multiple GBS genomes revealed only one chromosomal copy of the PGK gene (Glaser *et al.*, 2002;Tettelin *et al.*, 2002;Tettelin *et al.*, 2005). In most cases, the genes encoding glycolytic enzymes are essential genes, and therefore characterization of the role these proteins play in pathogenesis is difficult, as the traditional approach of creating an isogenic knock-out mutant is not possible. Thus, my goal in this chapter was to use alternative methods to characterize GBS PGK with respect to its role in pathogenesis.

The enzymatic activity of 200 U per mL of purified rGBS-PGK is similar in order of magnitude to values reported for other purified bacterial PGKs. For example, *Bacillus stearothermophilus* PGK has a reported activity of 557 U/mg protein (Suzuki and Imahori, 1982), and purified *E. coli* PGK an activity of 98 U/mg protein (D'Alession and Josse, 1975). PGK from other sources also exhibited similar activity; Lee *et al.* found PGK purified from *S. mansoni* possessed an activity of 250 U/mg protein, and rabbit PGK an activity of 500 U/mg protein (Lee *et al.*, 1995). Therefore the assayed enzyme activity of rGBS-PGK suggests that the addition of the 6XHis motif to GBS PGK does not hinder proper folding or enzymatic function, and is a viable approach to overexpression and purification of this protein.

In correlation with the previous finding that scPGK inhibited GBS invasion of epithelial cells (Burnham *et al.*, 2005), incubation of HeLa cells with rGBS-PGK prior to GBS infection inhibited GBS invasion. Additionally, it was found that scPGK need not be enzymatically active for inhibition of GBS invasion to occur. This suggested that PGK inhibition of GBS invasion is a result of the PGK protein interacting with the host cell, either directly by binding to a host cell moiety (a protein-protein interaction) or modulating host cell signal transduction processes involved in GBS invasion (or possibly both). One possibility is that the addition of exogenous PGK to HeLa cells prior to bacterial infection saturates PGK binding sites within the host cell, thereby obstructing the interaction between PGK on the GBS bacterial cell surface and host cell moieties during the invasion process.

In addition to enzymatic activity, various functions for PGK in other species have been reported. PGK on the surface of *Candida albicans* is a plasmin(ogen) binding protein (Crowe *et al.*, 2003), and PGK from rabbit muscle has the ability to bind actin (Arnold *et al.*, 1971;Bronstein and Knull, 1981). Overexpression of PGK in human bronchial epithelial cells resulted in decreased expression of urokinase-type plasminogen activator receptor (uPAR) at the surface of these cells; this is mediated by specific binding of PGK to the coding region of uPAR messenger RNA (mRNA) (Shetty *et al.*, 2004). Further, Wang and colleagues recently postulated that PGK can play a role in tumor invasion and growth (Wang *et al.*, 2007). This is based on their finding that PGK overproduction in tumor cells is followed by an increase in expression of molecules involved in cell-cell adhesion, such as β-catenin, and that PGK secreted by tumors has the ability to cleave plasmin (Wang *et al.*, 2007). Therefore, there are many functions for PGK in addition to its role in glycolysis. Further, this is not the first report of the binding properties of PGK being independent of enzymatic function. Shetty *et al.* created PGK clones (P204H and D219A) which possessed less than 20% of the wild-type PGK enzymatic activity; both mutant and wild-type PGK had the ability to bind to uPAR mRNA (Shetty *et al.*, 2005), illustrating that PGK regulated modulation of uPAR expression is independent of enzymatic activity.

In my previous study, it was observed that the addition of scPGK to HeLa cells resulted in modification of the actin cytoskeleton (Burnham *et al.*, 2005). At that time, it was unclear if this was mediated by PGK binding to a receptor on the host cell surface, possibly triggering a signal transduction cascade resulting in the cytoskeletal modification, or PGK inside the cell triggering the actin rearrangement. Fluorescence microscopy in conjunction with a GFP-PGK fusion protein was used to visualize PGK expressed within the HeLa cell and its effect on the HeLa cell cytoskeleton. Marked disorganization of actin microfilaments inside the HeLa cell was observed with these experiments. My previous studies showed that α-actinin antibodies bind to GBS PGK, suggesting the presence of an actin binding domain in the GBS PGK molecule (Burnham *et al.*, 2005). α-actinin is an actin bundling and cross-linking protein that assembles actin microfilaments into a tetragonal lattice. A possible explanation for the effect of GBS PGK on the cytoskeleton is that PGK is binding to sites on actin where α-actinin would usually bind, resulting in a disruption of actin organization or polymerization. GBS disruption of host cell cytoskeletal function could be advantageous to the bacteria as this may allow the GBS to induce phagocytosis into a non-phagocytic cell, or to resist phagocytosis by a professional phagocyte.

The PGK protein structure is executed as two independently folded, globular domains that are similar in size but divided by a deep cleft or hinge region (Watson *et al.*, 1982). The domains correspond with the C-terminal and N-terminal halves of the protein, with the exception of 10 residues on the C-terminus, which form an α-helix that interacts with the N-terminal domain. Modification of the C-terminus of GBS PGK by the addition of a hydrophobic peptide sequence abolished GBS PGK-mediated cytoskeletal disruptions. A previous investigation observed that deleting the 15 terminal amino acids of the C-terminus of PGK changed the structure of both domains; the N-terminal domain was radically modified and resembled a "molten globule" state, and the C-terminal domain was structurally altered (Mas *et al.*, 1995). This data suggested that native PGK conformation requires the presence of a complete, unaltered C-terminus.

The GFP-PGKTail construct did not induce the same cytoskeletal changes or GFP fluorescence pattern seen with GFP-PGK alone. Transfection of HeLa cells with GFP-PGKTail resulted in "bubbles" or "circles" of GFP fluorescence localized near the HeLa cell nucleus. One possible explanation for this observation is that hydrophobic domain of the recombinant GFP-PGKtail proteins may be interacting with each other, and aggregating inside the HeLa cell cytoplasm, forming the observed fluorescent "bubbles". A second scenario is that the placement of the hydrophobic peptide on the PGK C-terminus disrupts protein folding, perhaps in a fashion analogous to the "molten globule" state described for the PGK C-terminal deletion mutant (Mas *et al.*, 1995). A third explanation relates to the process of protein expression and processing in eukaryotic cells. In the eukaryotic cell, transcription takes place in the cell nucleus and then mRNA exits the nucleus into the cytoplasm, onto the ribosomes, which bind to the rough endoplasmic reticulum (RER), the site where most protein synthesis occurs. The RER is a compartment formed by a complex network of membranes where synthesized proteins collect for transport throughout the cell. In some cases, proteins from the RER are transported to the Golgi apparatus in vesicles formed by budding off of the RER. The Golgi is a stack of membrane-bound vesicles responsible for post-translational modification, sorting, and shipping of synthesized proteins. Thus, it is possible that the hydrophobic motif on PGK interacts with membrane components of the RER or Golgi, (and/or with the nuclear membrane) and becomes trapped in these cellular locations. The altered C terminus of the PGK protein may also interfere with normal sorting or trafficking of the expressed protein such that it is sequestered in the rough endoplasmic reticulum or Golgi apparatus of the host cell, and thus is not released into the cytoplasm where it could interact with the host cell cytoskeleton. This may be the cause of the altered GFP fluorescence pattern in GFP-PGKtail-expressing HeLa cells and the lack of actin cytoskeleton disruption observed in these cells compared to the cells expressing wild-type GFP-PGK.

The outer surface molecules of bacterial pathogens have served as the focus of numerous investigations, as these moieties often mediate crucial interactions at the host-pathogen interface (Baron *et al.*, 2004;Bergmann *et al.*, 2004;Hughes *et al.*, 2002;Navarre and Schneewind, 1999;Tamura *et al.*, 1994). In addition to their participation in virulence, surface molecules attract interest for their role as potential vaccine targets. Several examples exist in the literature of glycolytic enzymes decorating the outside surface of Streptococci (Bergmann *et al.*, 2001;Bergmann *et al.*, 2004;Hughes *et al.*, 2002;Pancholi and Fischetti, 1993;Pancholi and Fischetti, 1998;Winram and Lottenberg, 1996), a cellular compartment seemingly unrelated to the function of these proteins. However, these surface-associated "enzymes" have been demonstrated to participate in various facets of virulence in GAS and pneumococcus, and augment the invasive disease process in these organisms. In this chapter, I have expressed and purified one such enzyme, PGK, found on the surface of GBS. The addition of PGK to epithelial cells prior to GBS infection was found to be inhibitory to GBS invasion of these cells, although scPGK need not be enzymatically active for this effect to occur. Further, expression of GBS PGK inside the HeLa cell results in marked disruption of the actin cytoskeleton of these cells. Invasion of GBS is a complex and multifactorial process, and PGK located on the GBS surface appears to be an important virulence factor involved in cellular invasion by this organism.

Table 4.1. *E. coli* strains used in this investigation.

***E. coli* Strain**	**Relevant Description**	**Source**
JM101	Possesses a *lacI*^{q} mutation; used for propagation and storage of pQE30 plasmids	Laboratory Stocks
M15	Does not harbor chromosomal *lacI*^{q} mutation; used for high-level 6XHis protein expression	Qiagen
DH5α	A recombination-deficient strain used for cloning and plasmid propagation	Laboratory Stocks
XL-1 Blue	A recombination-deficient, high-efficiency cloning strain	Stratagene
TOP10	Suitable for TOPO® cloning	Invitrogen

Table 4.2. Plasmids used in this investigation.

Plasmid	Relevant Description	Reference or Source
pQE30	6XHis fusion protein expression vector; Amp^r	Qiagen
pQE30-PGK	pQE30 containing NCS13 PGK gene lacking the start codon; Amp^r	This work
pREP4	Constitutive expression of *lac* repressor protein; Kan^r	Qiagen
pFW11	pFW5 derivative; $Spectinomycin^r$	(Podbielski *et al.*, 1996)
pFW11-PGKB	pFW11 containing NCS13 PGK gene; $Spectinomycin^r$	This work
pFW11-PGKB-Tail	pFW11-PGKB with a hydrophobic tail sequence inserted at the C-terminus of PGK; $Spectinomycin^r$	This work
pcDNA3.1/NT-GFP-TOPO®	GFP fusion protein expression vector, Amp^r	Invitrogen
pGFP-PGK	pcDNA3.1/NT-GFP containing the NCS13 PGK gene lacking the start codon; Amp^r	This work
pGFP-PGKrev	pcDNA3.1/NT-GFP containing the NCS13 PGK gene (minus the start codon) in the antisense direction; Amp^r	This work
pGFP-PGKTail	pGFP-PGK with a hydrophobic tail sequence inserted at the C-terminus of PGK; Amp^r	This work
pEGFP-Actin	GFP fused to N terminal sequence of human cytoplasmic β-actin; Amp^r	Clontech

Table 4.3. Oligonucleotides used in this investigation.

Oligonucleotide Name	**Sequence**
PGK-Bam-His	5’ TTC**GGATCC**GCTAAATTGACTGTTAAA
PGK-Pst-His	5’ TTC**CTGCAG**TTATTTTTCAGTCAATGC
pQE30-Seq-For	5’ CGGATAACAATTTCACAGAG
pQE30-Seq-Rev	5’ GTTCTGAGGTCATTACTGG
Sal-PGK-New-R	5’ ACGCGTCGACATGGCTAAATTGACTGTT AAAGACGTT
Bam-PGK-New-F	5’ CGCGGATCCTTTTTCAGTCAATGCTGCCAA ACCTGG
HB-Tail-F2	5' GCATTGACTGAAAAAATTCTTTTCATTA TTGGTTTAATTATGCTTGCAGTAGTTATCATGT AAGGATCCTCG AGCTCT
HB-Tail-R2	5' AGAGCTCGAGGATCCTTACATGATAACTA CTGCAAGCATAATTAAACCAATAATGAAAAGA ATTTTTTCAGT CAATGC
PGK-SEQ-R-1	5’ TTGGCCGTGCTGACAAATTCTCATGGA
GFP-PGK-F	5’ GCTAAATTGACTGTTAAAGACGTTGATTTG
Bam-PGK-C-R	5’ GACGGATTCTTATTTTTCAGTCAATGCTGC CAA
PGK-Tail-Asc-R	5’ CGGCGCGCCTTACATGATAACTACTGCAA GCAT
GFP Forward	5’ CGACACAATCTGCCCTTTGC

a.

1 MAKLTVKDVDLKGKKVLVRVDFNVPLKDGVITNDNRITAALPTIKYIIEQGGRAILFSHL

61 GRVKEEADKEGKSLAPVAAD LAAKLGQDVVFPGVTRGAKLEEAINALEDGQVLLVENTRF

121 EDVDGKKESKNDEELGKYWASLGDGIFVNDAFGTAHRAHASNVGISANVEKAVAGFLLEN

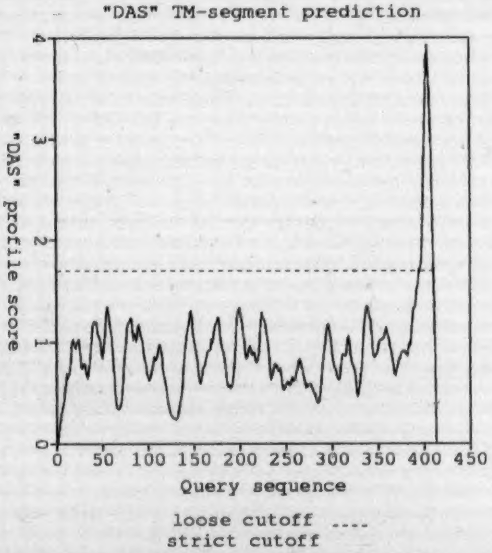
181 EIAYIQEAVETPERPFVAILGGSKVSDKIGVIENLLEKADKVLIGGGMTYTFYKAQGIEI

241 GNSLVEEDKLDVAKDLLEKSNGKLILPVDS KEANAFAGYTEVRDTEGEAVSEGFLGLDIG

301 PKSIAKFDEALTGAKTVVWNGPMGVFENPDFQAGTIGVMDAIVKQPGVKSIIGGGDSAAA

361 AINLGRADKFSWISTGGGASMELLEGKVLPGLAALTEK **ILFIIGLIMLAVVIM**

b.



http://www.sbc.su.se/~miklos/DAS/

Fig. 4.1. Strategy for the insertion of a hydrophobic peptide at the C-terminus of GBS PGK. (a). The amino acid sequence of GBS PGK (Tettelin *et al.*, 2002), with the inserted hydrophobic tail on the C-terminus illustrated by bold print. The hydrophobic tail is derived from the "cell wall surface anchored family protein" (SAG1407) (Tettelin *et al.*, 2002) that has been modified slightly to obtain an optimal Dense Alignment Score (DAS). (b). DAS of GBS PGK with the added C-terminal hydrophobic tail sequence. No transmembrane segments are predicted in the wild-type PGK segment but the addition of the hydrophobic peptide tail sequence to the C-terminus results in a DAS with a hydrophobicity index within the predicted transmembrane value range.

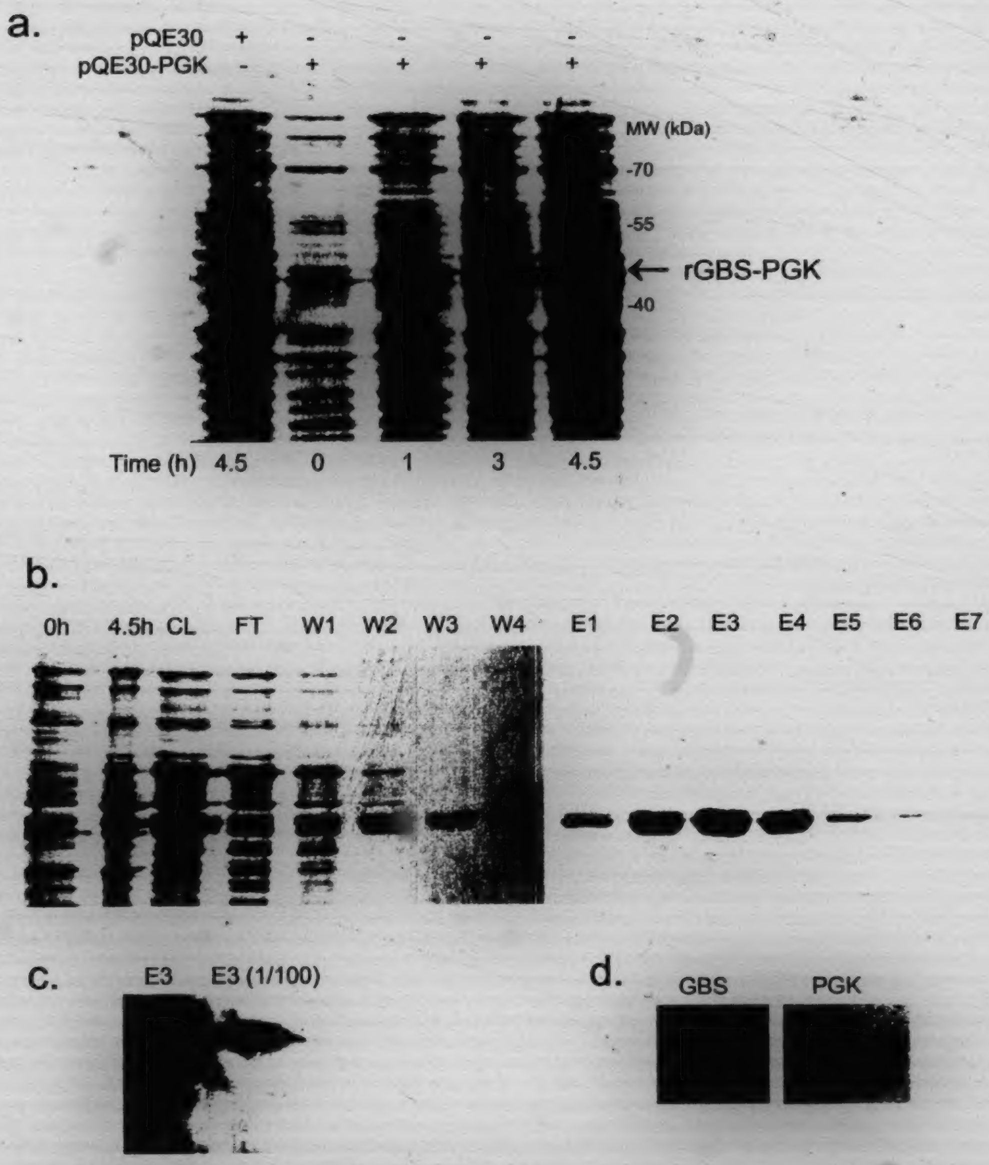

a.
pQE30 + - - - -
pQE30-PGK - + + + +
MW (kDa)
-70
-55
rGBS-PGK
-40
Time (h) 4.5 0 1 3 4.5
b.
0h 4.5h CL FT W1 W2 W3 W4 E1 E2 E3 E4 E5 E6 E7
c.
E3
E3 (1/100)
d.
GBS
PGK

Fig. 4.2. Expression and purification of rGBS-PGK in *E. coli* M15. (a). Time course of rGBS-PGK expression in M15. M15[pREP4][pQE30] was induced with 2 mM IPTG for 4.5 h and M15[pREP4][pQE30-PGK] was induced with 2 mM IPTG for 0, 1, 3, or 4.5 h. Crude whole-cell lysates were resolved using 10% SDS-PAGE, and stained with Coomassie Blue. (Continued next page).

Fig. 4.2 (continued). (b). Ni-NTA purification of 6XHis-PGK (rGBS-PGK) produced in M15. The cleared lysate of M15 expressing rGBS-PGK was passed through an Ni-NTA affinity column, and eluted in a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole (pH 8.0). Proteins were resolved by 10% SDS-PAGE and stained with Coomassie Blue. 0 h, whole-cell M15[pREP4][pQE30-PGK] uninduced lysate; 4.5 h, whole-cell M15[pREP4][pQE30-PGK] lysate at 4.5 h post IPTG induction; CL, cleared lysate; FT, Ni-NTA column flow-through; W1-W4, wash fractions 1-4 from the Ni-NTA column; E1-E7, soluble rGBS-PGK elutate fractions 1 through 7 from the Ni-NTA column. (c). Western blot of anti-polyhistidine antibody binding to rGBS-PGK. Eluate fraction 3 (E3) and E3 diluted 1/100 were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-polyhistidine antibody. (d). Western blot of anti-α-actinin antibodies binding to PGK. Crude whole-cell lysate of GBS NCS13 (GBS) or purified rGBS-PGK (PGK) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-α-actinin antibody.

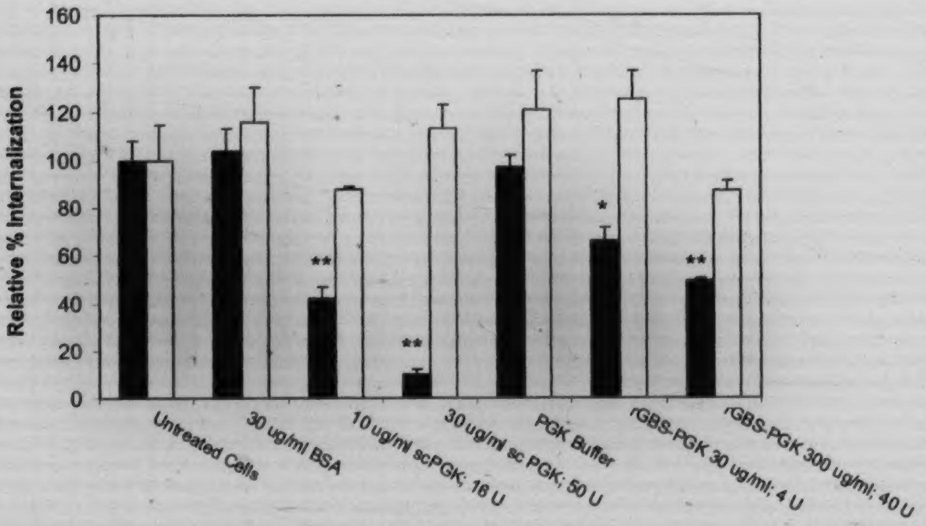


Fig. 4.3. The addition of rGBS-PGK to HeLa cells prior to GBS infection inhibits GBS invasion. HeLa cells were either left untreated, or treated with 30 μg/mL BSA, 10 μg/mL scPGK, 30 μg/mL scPGK, PGK buffer, 30 μg/mL rGBS-PGK, or 300 μg/mL rGBS-PGK for 1 h prior to infection with GBS (■; solid bars) or *S. typhimurium* (□; open bars), and an antibiotic protection invasion assay was performed. Relative percent internalization was calculated as follows: [(Number of CFU recovered from treated cells/Number of CFU recovered from untreated control cells) X 100%]. The graphical representation illustrates one representative experiment in which each assay condition was performed in triplicate. Error bars represent standard error of the mean. "*" indicates a *P* value < 0.05 and "**" a *P* value of < 0.01 compared to invasion into untreated control cells, as evaluated using Dunnet's Multiple Comparisons Test.

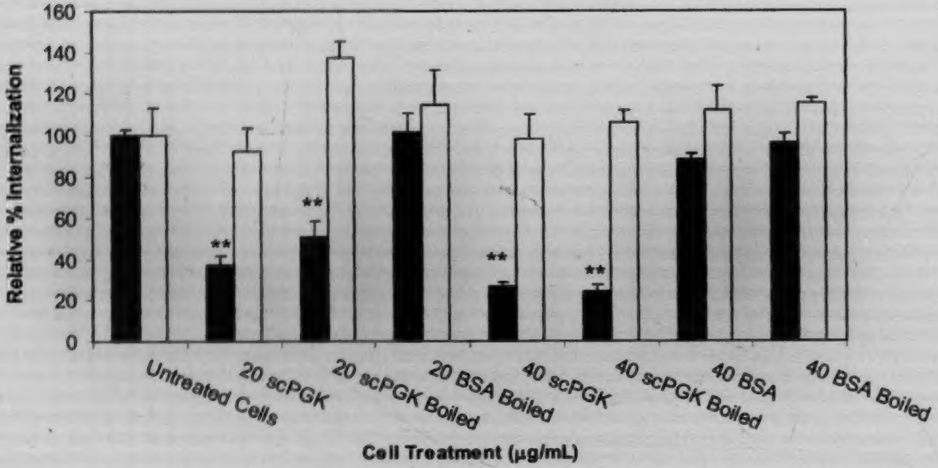


Fig. 4.4. Boiled scPGK inhibits GBS invasion. HeLa cells were either left untreated, or treated with BSA, scPGK, or boiled scPGK (20 µg/mL or 40 µg/mL) for 1 h prior to infection with GBS (■; solid bars) or *S. typhimurium* (□; open bars) and an antibiotic protection invasion assay was performed. Relative percent internalization was calculated as follows: [(Number of CFU recovered from treated cells/Number of CFU recovered from untreated control cells) X 100%]. The graphical representation illustrates one representative experiment in which each assay condition was performed in triplicate. Error bars represent standard error of the mean. "**" indicates a P value of < 0.01 compared to invasion into untreated control cells, as evaluated using Dunnet's Multiple Comparisons Test.

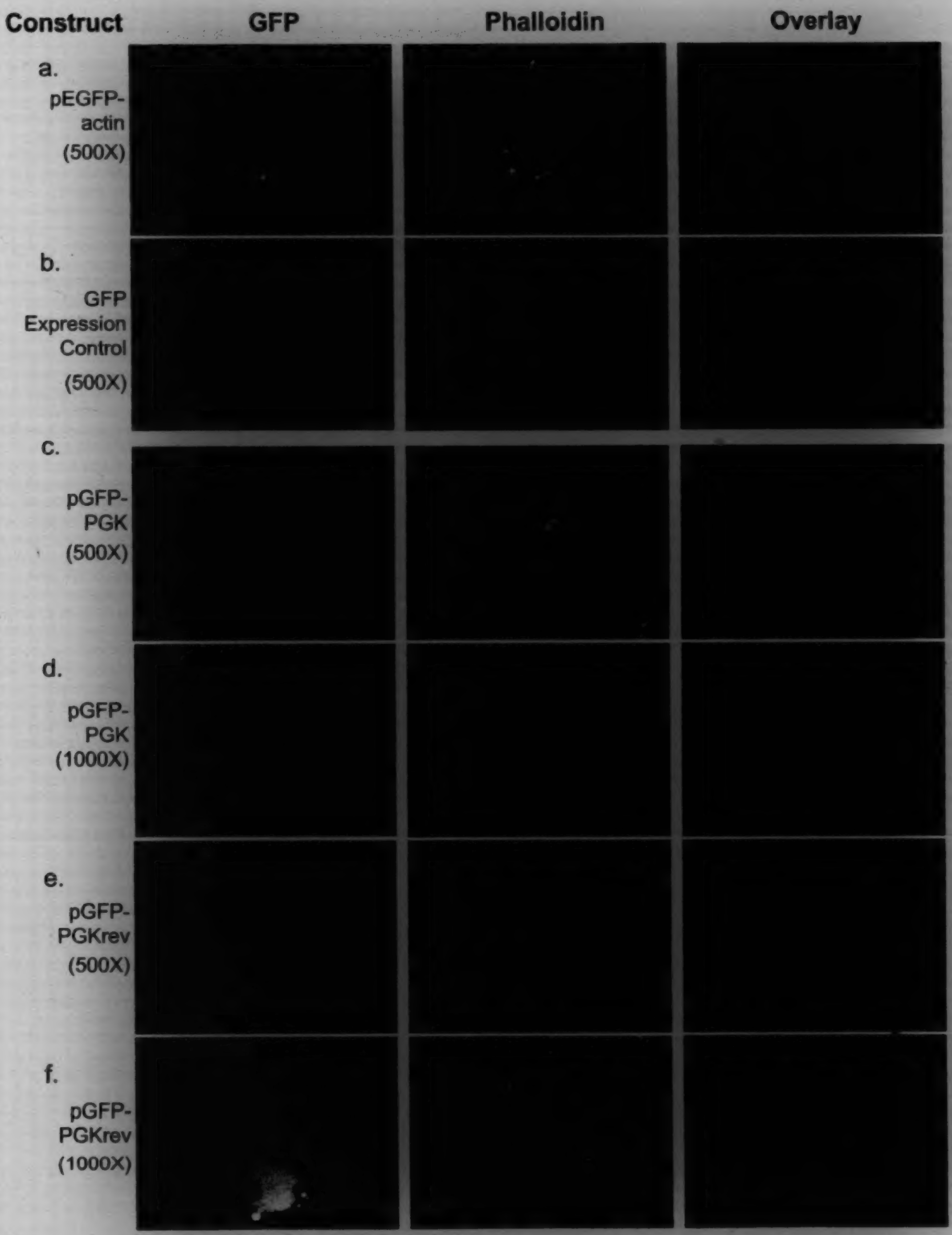

Construct
GFP
Phalloidin
Overlay
a.
pEGFP-actin
(500X)
b.
GFP Expression Control
(500X)
c.
pGFP-PGK
(500X)
d.
pGFP-PGK
(1000X)
e.
pGFP-PGKrev
(500X)
f.
pGFP-PGKrev
(1000X)

Fig. 4.5. Expression of GBS PGK inside the HeLa cell (continued next page).

Fig. 4.5 (continued). Expression of GBS PGK inside the HeLa cell disrupts the actin cytoskeleton. HeLa cells were transfected with Green Fluorescent Protein (GFP) expressing plasmids fused to either actin (a), an expression control expressing only GFP (b), GFP-GBS-PGK (c and d) or GFP-GBS-PGKrev (e and f). Cells were fixed, permeabilized, and stained with Alexa Fluor 568-labeled phalloidin to detect actin microfilaments and Hoescht Dye to detect cellular nuclei, and then visualized using fluorescence microscopy. The actin cytoskeleton is altered in GFP-GBS-PGK expressing cells; the stress fiber pattern is disrupted, and F-actin is largely restricted to the periphery of the cells. This is not observed in HeLa cells expressing the other constructs. The overlay column depicts GFP, phalloidin and Hoescht fluorescence. 500X and 1000X refer to magnification of the sample.

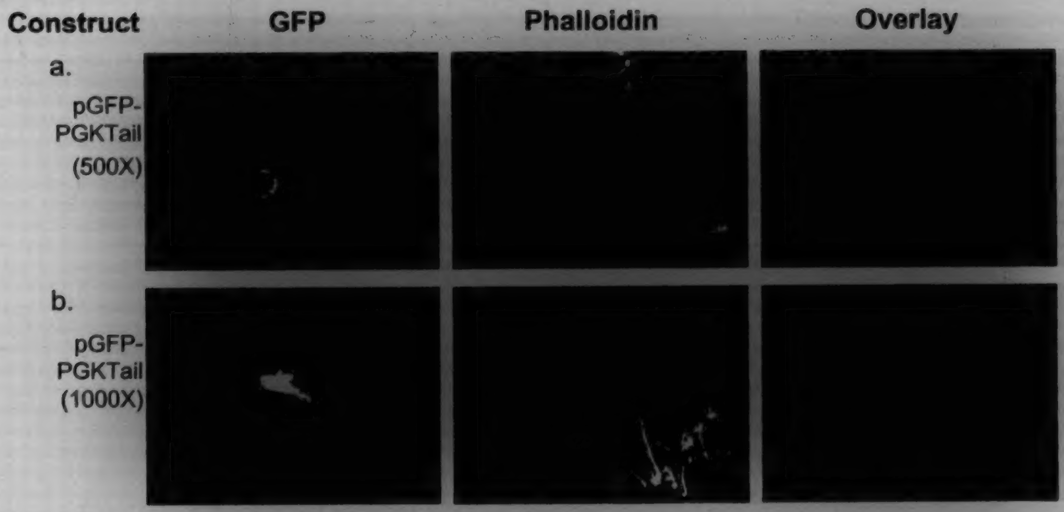


Fig. 4.6. The addition of a hydrophobic peptide sequence to the C-terminus of GBS PGK prior to expression in the HeLa cell abolishes cytoskeletal modifications observed in response to PGK expression. HeLa cells were transfected with Green Fluorescent Protein (GFP) expressing plasmids fused to PGK, modified to include a hydrophobic peptide sequence on the C-terminus (pGFP-PGKTail). Cells were fixed, permeabilized, stained with Alexa Fluor 568-labeled phalloidin to detect actin microfilaments and Hoescht Dye to detect cellular nuclei, and then visualized using fluorescence microscopy. In contrast to the cells expressing GFP-GBS-PGK (Fig. 4.5), the GFP-GBS-PGKTail expression pattern inside the HeLa cell is largely contained in a peri-nuclear halo, forms a lace-like pattern, and does not reach the periphery of the cells. No disruption in the actin cytoskeleton of the HeLa cell is observed in the cells expressing the mutant PGK. The overlay depicts GFP, phalloidin, and Hoescht fluorescence.

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Chapter 5:

Rac1, RhoA, and Cdc42 Participate in HeLa Cell Invasion by Group B *Streptococcus*

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5.1 Introduction

For a bacterial pathogen, the ability to invade non-phagocytic host cells presents a number of advantages, such as the ability to overcome unfavorable host conditions, become inaccessible to the host's immune system or gain access to privileged sites within the host. To augment the invasion process, bacterial pathogens have evolved mechanisms to interact with host cells by usurping or parasitizing existing host-cell machinery and signaling cascades.

The Rho-family GTPases are ubiquitous small GTPases that function as "molecular switches" within eukaryotic cells that interact with a diverse array of downstream effector molecules, and control signal transduction pathways by linking membrane events to cytoskeletal modulation (Fig. 5.1). Manipulation of host cell signaling pathways by an invasive pathogen can be a key event in bacterial survival and dissemination and therefore Rho GTPases are major targets for virulence factors of several bacterial pathogens. C3 exoenzyme was the first bacterial protein demonstrated to interfere with functionality of Rho GTPases (Aktories *et al.*, 1987), and since then, many other bacterial factors have been identified that interact with this family of molecules, either directly or indirectly, and as activators or inhibitors.

The Group B *Streptococcus* (GBS) is an important human pathogen with the ability to cause invasive disease. In the Western world, GBS is the leading cause of meningitis in infants less than one month of age (Schuchat *et al.*, 1997). This is believed to be a consequence of passage of the organism to the infant during delivery by a colonized mother (Schuchat, 1998;Tyrrell *et al.*, 2000). In addition to meningitis, GBS infections in infants can include pneumonia, sepsis, and soft tissue infections. GBS serotypes Ia, Ib, and III are most strongly associated with neonatal infections. Invasive GBS infections are not reserved for infancy, but also occur in adulthood, especially in the elderly or in those individuals with an underlying chronic disease, such as diabetes mellitus (Farley *et al.*, 1993;Schuchat *et al.*, 1990;Schuchat, 1998;Tyrrell *et al.*, 2000). Serotype V is a major cause of GBS infection in adulthood, especially in North America. Disease manifestations of invasive GBS infection in adults include meningitis, pneumonia, sepsis, and infections of bone and soft tissue.

In order to cause infection, GBS must be able to subvert a variety of host cell types, such as host epithelial cells, which are a major component of innate immunity and, in many cases, pose as the host's first line of defense against bacterial assault. There have been numerous investigations focused on the invasion of bacterial pathogens into host cells in recent years. While it has been well documented that GBS are able to invade a variety of cell types, including epithelial and endothelial cells (Gibson *et al.*, 1993;Lalonde *et al.*, 2000;Shin and Kim, 2006;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997), the specific molecular mechanisms involved in this process remain relatively uncharacterized. What has been established, however, is that host cell actin microfilaments are recruited during the process of GBS attachment and subsequent invasion. This was demonstrated by showing that cytochalasin D, a potent inhibitor of actin polymerization, inhibits GBS invasion into epithelial cells (Gibson *et al.*, 1993;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997), and that actin is recruited to the site of GBS attachment (Tyrrell *et al.*, 2002).

The Rho-family GTPases are obvious candidates for participation in GBS-induced actin manipulation, as it is well established that activation of these moieties is involved in actin nucleation and polymerization, leading to the alteration or formation of cytoskeletal structures within the host, such as actin bundling (Fig 5.1). Consequences of these events include formation of membrane protrusions, phagocytosis, vesicle trafficking and cytokinesis. The objective of this chapter was to evaluate the role of the Rho-family GTPases Rac, Rho, and Cdc42 in GBS invasion into epithelial cells. Chemical modulators of these GTPases as well as dominant negative forms of Rac, Rho, and Cdc42 were used in conjunction with an antibiotic protection invasion assay to address this objective.

5.2. Materials and Methods

5.2.1. Bacterial strains, cell lines, and growth conditions.

The serotype V GBS strain NCS13 was isolated from the soft tissue wound of an elderly patient, and has been previously described (Tyrrell *et al.*, 2002). The *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) control strain NCTC 10241 was obtained from the National Culture Collection, PHLS Colindale, London, England. These organisms were cultured at 35 °C on sheep blood agar plates (BAPs) (Dalynn Biologicals) or in Todd-Hewitt Broth (THB) (Difco Laboratories).

The human epithelial cell line HeLa 229 (ATCC CCL-2.1) was obtained from the American Type Culture Collection (ATCC) and was grown in Opti-MEM I reduced serum medium supplemented with 4% fetal bovine serum (FBS) (Gibco).

5.2.2. Chemical inhibitors.

Compactin (mevastatin), Y-27632 dihydrochloride, L-α-lysophosphatidic acid (LPA), *Clostridium difficile* Toxin B, and ADP-ribosyltransferase C3 were purchased from Sigma-Aldrich. HeLa cells were treated with compactin for 18 h and Toxin B for 3 h prior to infection with bacteria. For treatment with exoenyzme C3, the cells were incubated for 18 h in the presence of 1 μg/mL Lipofectamine™ 2000 (Invitrogen), a modification of the previously described Lipofectamine procedure (Borbiev *et al.*, 2000). HeLa cells were incubated with the other inhibitors for 1 h prior to bacterial infection of the cells. The inhibitors were present during the invasion assay until the first wash when the fresh media with antibiotics was added.

5.2.3. Invasion (antibiotic protection) assay.

Invasion of bacteria into epithelial cell cultures was quantified using a standard antibiotic protection invasion assay with minor modifications (Rubens *et al.*, 1992;Tyrrell *et al.*, 2002); this assay has been used extensively to investigate the streptococcal invasion process (Ozeri *et al.*, 2001;Purushothaman *et al.*, 2003;Shin and Kim, 2006;Valentin-Weigand *et al.*, 1997). Briefly, HeLa cell monolayers were grown to confluence in 24 well plates, and then treated with chemical inhibitors. GBS or *S. typhimurium* were grown in THB overnight. A 0.5 McFarland standard of organism was made in THB and then diluted 1 in 100 in THB. For GBS, 100 µL of this suspension (approximately 1.5 X 10^5 bacteria) and for *S. typhimurium* 25 µL of this suspension (approximately 4 X 10^4 bacteria) was applied to the monolayer. The difference in inoculum is reflective of the fact that *S. typhimurium* invades epithelial cells with higher efficiency than GBS. Following inoculation, the plates were centrifuged at 100 *g* for 5 min at room temperature. After incubating at 35 °C for 2 h to allow bacterial internalization, the monolayer was washed three times with phosphate buffered saline (PBS) to remove unbound bacteria. Any bacteria that had bound but not internalized were killed by incubation for 2 h with fresh media containing 5 µg of penicillin per mL and 100 µg of gentamicin per mL. After the 2 h incubation, an aliquot of media was applied to a BAP to ensure that the antibiotic treatment was effective and all extracellular bacteria were successfully killed. The monolayers were washed with PBS, trypsinized, and then lysed with 0.1% Triton X-100. The lysates were spread onto BAPs and incubated overnight at 35 °C. The number of colonies on each plate was counted to determine the number of CFU that had invaded the cell monolayer. For the "zero" concentration for each inhibitor, the solvent for that inhibitor (eg. DMSO) was added to the cells at a concentration equal to the solvent concentration present during the inhibitor treatment (control cells).

The integrity of the epithelial cell monolayer following inhibitor treatment was evaluated with microscopy. The cells were examined at the end of each incubation period during the assay to observe any morphological changes induced by the inhibitors. Portions of the HeLa cell culture supernatants were plated on to BAPs following the 2 h infection time with GBS and *S. typhimurium* to ensure that the inhibitor treatments were not resulting in reduced bacterial viability.

5.2.4. Transient transfection of HeLa cells.

Plasmids expressing dominant negative Rac (pcDNA3-EGFP-Rac1-T17N), Rho (pcDNA-EGFP-RhoA-T19N) and Cdc42 (pcDNA-EGFP-Cdc42-T17N) were provided by Dr. Garry Bokoch (The Scripps Research Institute, La Jolla, CA) (Ben-Ami *et al.*, 1998;Subauste *et al.*, 2000;Zhang *et al.*, 1995). For transient transfection assays, HeLa cells were grown to approximately 80-90% confluence in 24 well culture dishes. The culture media was replaced with serum-free Opti-MEM I, and 1 μg of plasmid DNA and 2 μg of Lipofectamine^{TM} 2000 Transfection Reagent (Invitrogen Life Technologies, Burlington, ON, Canada) were added to the cells according to the manufacturer's instructions. After 5 h incubation, the cell culture media was replaced with fresh Opti-MEM I with 4% FBS and the cells were incubated overnight at 37 °C.

A subset of transfected cells were grown on glass coverslips in the 24 well plates. Hoechst Dye (Sigma-Aldrich) was used to stain cell nuclei prior to visualization of the coverslips by fluorescence microscopy (Olympus Microscopes, Carsen Group) to evaluate transfection efficiency. The proportion of Green Fluorescent Protein (GFP) expressing cells was visually compared to the total cell population.

5.2.5. Statistical analysis.

For the invasion assays, relative percent internalization was calculated as follows: [(Number of CFU invaded into treated cells/Number of CFU invaded into control cells) X 100%]. GraphPad Instat 3 software was used for statistical analysis of the data; Dunnet's Multiple Comparisons Test was used to compare the invasion into inhibitor-treated or transfected cells to invasion into untreated control cells. A P value <0.05 compared to control cells was considered statistically significant and is designated by the character "*" on the graphical representations of the data. Each assay was performed at least 3 times and the graphical representations are illustrative of the mean of one representative experiment in which each assay condition was performed in triplicate. Error bars represent standard error of the mean (SEM).

5.3. Results

5.3.1. The pan-GTPase inhibitor Compactin blocks GBS uptake into epithelial cells.

Compactin is a pan-GTPase inhibitor that prevents post-translational modifications essential for GTPase function, such as Rho geranylgeranylation (Seabra, 1998). Compactin was used to screen for potential involvement of GTPases in the GBS internalization process. HeLa cell monolayers were treated with compactin for 18 h prior to GBS infection. Compactin had a potent inhibitory effect on GBS invasion, with over 90% reduction in internalization seen at a concentration of 10 μM (Fig. 5.2).

5.3.2. *Clostridium difficile* Toxin B inhibits GBS internalization.

The Rho family GTPases regulate the organization and assembly of the actin cytoskeleton in all eukaryotic cells (Burridge and Wennerberg, 2004;Hall, 1998). The strong inhibitory effect of compactin treatment on GBS invasion further suggested Rho-family GTPases could be involved in this process. To investigate this possibility, HeLa cells were treated with Toxin B (ToxB) from *C. difficile*. ToxB inactivates Rac, Rho, and Cdc42 by monoglucosylating these moieties in the GTPase binding pocket, thereby rendering them inactive (Aktories, 1997). ToxB inhibited GBS internalization in a dose dependant manner with a 50% reduction in invasion occurring at 100 ng/mL (Fig. 5.3). This is similar to what is observed for *S. typhimurium*, a pathogen whose invasion has been reported to be inhibited by ToxB (Criss and Casanova, 2003).

5.3.3. Chemical inhibition of Rho results in reduced GBS invasion.

C. botulinum C3 exoenzyme is an ADP ribosyltransferase that inactivates Rho, resulting in redistribution of the actin cytoskeleton due to interferences of Rho interaction with its effectors (such as Rho kinase). As a consequence, changes in cellular morphology are observed, such as cell rounding (Aktories, 1997). In contrast to many other bacterial toxins, C3 exoenzyme lacks a membrane binding and/or transfer component and thus cannot permeate the cell cytoplasm independently to reach its effectors. Hence, lipofectamine reagent needs to be used in concert with the C3 exoenzyme to permit toxin entry into the host cell (Borbiev *et al.*, 2000). HeLa cells were treated with lipofectamine, C3 exoenzyme, or both for 18 h prior to GBS infection. Treatment of the HeLa cells with C3 exoenzyme or lipofectamine alone did not inhibit GBS invasion (Fig. 5.4a). However, the C3 exoenzyme/lipofectamine combination treatment inhibited GBS invasion by 50% (Fig. 5.4a), suggesting that Rho specifically is involved in GBS invasion.

5.3.4. Activation of Rho leads to increased GBS invasion.

Lipophosphatidic acid (LPA) is a growth factor that activates Rho, leading to the formation of stress fibres and focal adhesions (Hall, 1998;Meerschaert *et al.*, 1998;Tapon and Hall, 1997). Since the inhibition experiments suggested that Rho is involved in GBS invasion, I wanted to investigate if the activation of Rho by

LPA would have an effect on the GBS internalization process. At a concentration of 50 μM, LPA increased GBS invasion of HeLa cells by nearly 50% (Fig. 5.4b). In contrast, *S. typhimurium* invasion was unaffected by the LPA treatment (Fig. 5.4b).

5.3.5. Inactivation of Rac1, RhoA, and Cdc42 inhibits GBS invasion into epithelial cells.

To further investigate the individual role of Rho-family GTPases, dominant negative forms of these moieties were used. HeLa cells were transiently transfected with the dominant negative form of Rac1 (Rac1-T17N), RhoA (RhoA-T19N) or Cdc42 (Cdc42-T17N). Each of these constructs was fused with GFP to allow for microscopic evaluation of the transfection efficiency, which varied from approximately 60 to 70%. *S. typhimurium* was assayed in parallel as a control. As expected, the internalization of *S. typhimurium* was reduced in the cells expressing the dominant negative form of Rac1 and Cdc42, but not RhoA (Fig. 5.5) (Chen *et al.*, 1996;Criss and Casanova, 2003;Lesser *et al.*, 2000). In contrast, the internalization of GBS was reduced in each of the transfected cell types examined. Rac1-T17N resulted in 75% reduction in GBS invasion, Cdc42-T17N a 51% reduction and RhoA-T19N a 42% reduction in comparison to control HeLa cells (Fig. 5.5). This confirms the results of the chemical inhibitor studies, and suggest that Rac, Rho, and Cdc42 are each involved in GBS invasion into host epithelial cells.

5.3.6. Inhibition of the Rho effector protein Rho-Kinase (ROCK) inhibits GBS internalization.

Rho-Kinase (ROCK) is a downstream Rho effector (Burridge and Wennerberg, 2004;Maekawa *et al.*, 1999), and has been implicated in Rho-mediated actin reorganization (Ohashi *et al.*, 2000). Y27632 dihydrochloride is a highly potent and specific inhibitor of ROCK. Invasion of HeLa cells by *S. typhimurium*, which invades independently of Rho (Criss and Casanova, 2003) was not reduced by Y27632 treatment (Fig. 5.6). Treatment of HeLa cells with Y27632 prior to GBS infection inhibited invasion in a dose dependant manner, with 30% inhibition at 5 μM and 65% inhibition at 20 μM (Fig. 5.6).

5.4. Discussion

Although GBS colonize some individuals asymptomatically, they are also capable of causing invasive disease. While it has been well established that GBS are able to invade epithelial cells, and that this contributes to the GBS disease process, the specifics of GBS cellular invasion remain largely uncharacterized. Previous studies have demonstrated that actin manipulation is a critical event in GBS invasion into host cells (Gibson *et al.*, 1993;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997). The goal of this chapter was to examine the cellular processes leading up to and involved in host cell actin manipulation by this pathogen.

The Rho-family GTPases are a set of "molecular switches" that have been studied extensively and are implicated in multiple facets of cytoskeletal regulation (Fig 5.1). The role of the Rho GTPases in bacterial pathogenesis has been widely investigated. Although one or more members of this family of GTPases may participate in the bacterial invasion process, there is great variation between different pathogens in regards to the kinetics of activation, protein binding partners, and signal transduction cascades involved. The results of this study illustrate that Rac, Rho, and Cdc42 each contribute to efficient invasion of HeLa cells by GBS.

It appears that the utilization of Rho GTPases by a bacterial pathogen to invade host cells can vary depending on the cell type. For example, in a study exploring RhoA and Rac1 in GBS invasion into human brain microvascular endothelial cells (HBMEC), Shin and Kim observed that GBS exhibit reduced internalization into HBMEC expressing dominant negative Rac1 and RhoA (Shin and Kim, 2006), but dominant negative expression of Cdc42 in HBMEC cells did not reduce GBS invasion as it did in HeLa cells. My finding that the ROCK inhibitor Y27632 reduced GBS internalization into HeLa cells points to ROCK as a downstream Rho effector in the GBS invasion process of these cells. In contrast, Y27632 treatment prior to infection does not reduce GBS invasion into HBMEC cells (Shin and Kim, 2006). This suggests that different downstream Rho effector proteins may be recruited or manipulated during endothelial and epithelial cell invasion.

It is interesting to note that the effect of ToxB on GBS invasion is not as potent as the reported effect on Group A Streptococcal (GAS) internalization.

With GAS, 5 ng/mL ToxB reduces GAS invasion into HeLa cells by over 90% (Ozeri *et al.*, 2001) whereas 100 ng/mL was required to achieve a 50% reduction in invasion for GBS. The finding that RhoA-T19N inhibits GBS invasion is of particular interest, as this is in contrast with investigations by Ozeri and colleagues who observed that dominant negative expression of RhoA in MDCK cells resulted in a marked increase in GAS internalization (Ozeri *et al.*, 2001). This finding could suggest a possible point of difference in host cell cytoskeleton exploitation and host cell signal transduction pathways utilized by these two organisms for invasion.

Throughout the literature, involvement of RhoA in the invasion process seems to best relate to the "zipper mechanism" of bacterial internalization, whereby engulfment of bacteria into the host cell does not require large membrane extensions. The invasion of *Brucella abortus* into HeLa cells is an example of this; the invasion of this pathogen requires Rho (as well as Rac and Cdc42), but occurs with cytoskeletal rearrangments that are visually subtle (Guzman-Verri *et al.*, 2001). This is in contrast to pathogens with Type 3 or 4 Secretion Systems, where the pathogen injects effector proteins into the host cell cytoplasm that interfere with GTPases directly, with Rac and Cdc42 being the most common targets (as is the case for *S. typhimurium*). This process tends to lead to drastic, dramatic, localized actin manipulations and membrane ruffling, termed "trigger" type phagocytosis. The involvement of RhoA in GBS invasion of HeLa cells demonstrated in this investigation, and its relation to "zipper-type" phagocytosis correlates with previous findings using TEM microscopy to examine GBS invasion of HeLa cells, whereby the host cell membrane appears to wrap tightly around the chains of GBS during the invasion process (Tyrrell *et al.*, 2002).

In the eukaryotic cell, Rho GTPases link stimuli to cytoskeletal plasticity by directing actin rearragment in the cell. It is still unclear at this time precisely how GBS triggers activation of Rho GTPases or what binding partners Rho GTPases may interact with in response to GBS invasion; this is a topic for future investigation. Rho GTPases also have the ability to activate and regulate one another; interplay at this level could also be an essential component of the GBS invasion process.

In conclusion, using the HeLa cell culture model, it was found that Rac, Rho, and Cdc42 each participate in GBS invasion into epithelial cells. Rac, Rho, and Cdc42 link extracellular signals to the formation of stress fibers, lamellipodia, or filpodia, respectively (Fig 5.1) (Burridge and Wennerberg, 2004;Hall, 1998;Tapon and Hall, 1997). The involvement of the Rho-family GTPases in GBS invasion may indicate that these cellular structures participate in GBS invasion of into epithelial cells, or may act synergistically to promote GBS uptake.

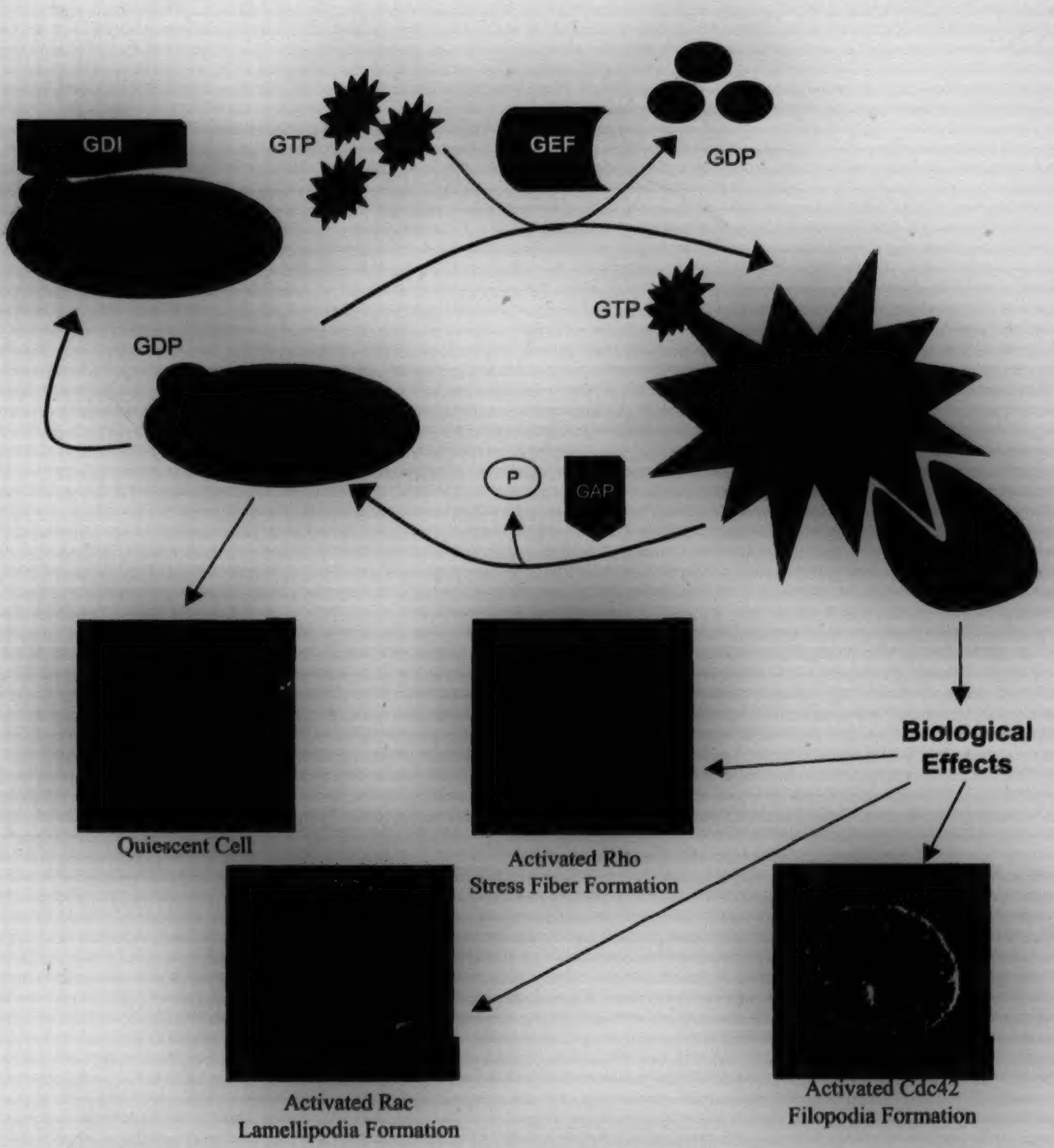
GDI
GTP
GEF
GDP
GTP
GDP
P
GAP
Biological Effects
Quiescent Cell
Activated Rho
Stress Fiber Formation
Activated Rac
Lamellipodia Formation
Activated Cdc42
Filopodia Formation

Fig. 5.1. The Rho-Family GTPases Rac, Rho, and Cdc42 regulate the actin cytoskeleton. GTPases cycle between the inactive, GDP-bound form, and the active, GTP-bound form. GTPases become activated by Guanosine Nucleotide Exchange Factors (GEFs) that catalyze nucleotide exchange by facilitating the release of GDP from the GTPase, followed by replacement with GTP. The rate of GTP hydrolysis is regulated by the GTPase Activating Proteins (GAPs). GTPases are also regulated by Guanosine Nucleotide Dissociation Inhibitors (GDIs), which bind to the GDP-bound form and prevent nucleotide exchange, maintaining the GTPase in the inactive state. Micrographs illustrate Swiss 3T3 fibroblast cells stained with rhodamine-labelled phalloidin to illustrate cytoskeletal changes observed in response to Rac, Rho, or Cdc42 activation in these cells (micrographs adapted from Hall, 1998).

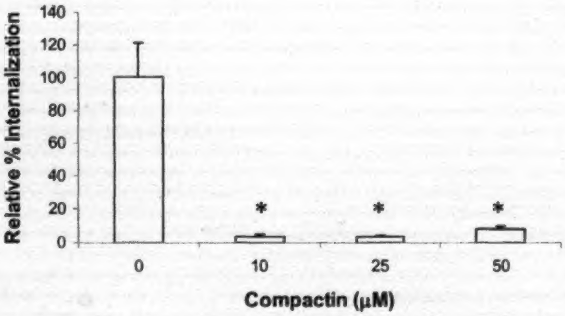

Fig. 5.2. Compactin, a pan-GTPase inhibitor, prevents GBS internalization. HeLa cells were treated with compactin for 18 h prior to GBS infection. Relative percent internalization was calculated as follows: [(Number of CFU invaded into cells with inhibitor treatment/Number of CFU invaded into control cells) X 100%]. Error bars represent standard error of the mean (SEM). * $P < 0.05$ compared to control cells.

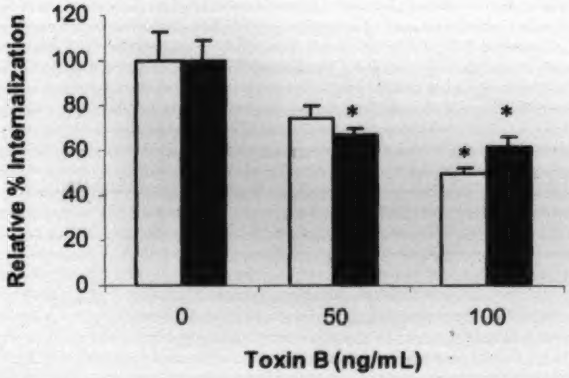

Fig. 5.3. Toxin B inhibits GBS internalization. Toxin B, which inhibits Rac, Rho and Cdc42 was used to treat HeLa cells for 3 h prior to infection. Both GBS and *S. typhimurium* exhibited dose dependant inhibition of invasion in response to the treatment. ■ *S. typhimurium*; □ GBS. Relative percent internalization was calculated as follows: [(Number of CFU invaded into cells with inhibitor treatment/Number of CFU invaded into control cells) X 100%]. Error bars represent standard error of the mean (SEM). * $P < 0.05$ compared to control cells.

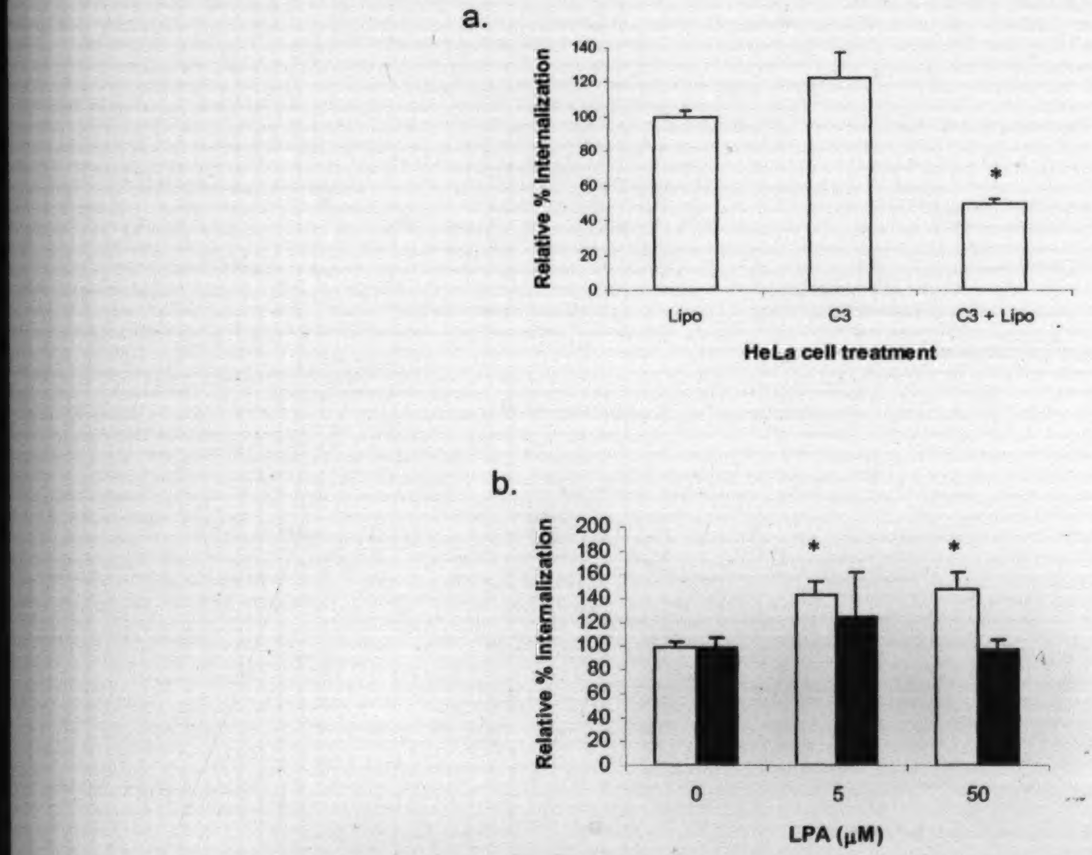

Fig. 5.4. Rho is involved in GBS invasion of HeLa cells. (a). HeLa cells were treated with either 1 μg/mL lipofectamine, 2 μg/mL exoenzyme C3, or both for 18 h prior to GBS infection. C3 exoenzyme treatment alone did not reduce GBS internalization, but the combination treatment of C3 with lipofectamine resulted in 50% inhibition of internalization. (b) HeLa cells were treated with lipophosphatidic acid (LPA), a growth factor that activates Rho, for 1 h prior to infection. The treatment increased GBS invasion but had a minimal effect on *S. typhimurium* invasion. ■ *S. typhimurium*; □ GBS. Relative percent internalization was calculated as follows: [(Number of CFU invaded into cells with inhibitor treatment/Number of CFU invaded into control cells) X 100%]. Error bars represent standard error of the mean (SEM). * $P < 0.05$ compared to control cells.

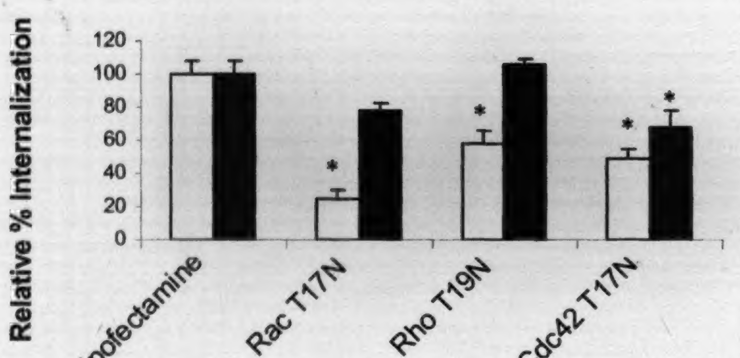

Fig. 5.5. Inactivation of Rac, Rho, and Cdc42 indicates these moieties are involved in GBS invasion. HeLa cells were transiently transfected with a dominant negative form of Rac1, RhoA, or Cdc42 prior to bacterial infection. GBS invasion is reduced in each of these cell types, while *S. typhimiurium* invasion is reduced in Rac1 and Cdc42 dominant negative genetic background but is not affected by dominant negative RhoA. ■ *S. typhimurium*; □ GBS. Relative percent internalization was calculated as follows: [(Number of CFU invaded into transfected cells/Number of CFU invaded into untreated cells) X 100%]. Error bars represent standard error of the mean (SEM). * $P < 0.05$ compared to control cells.

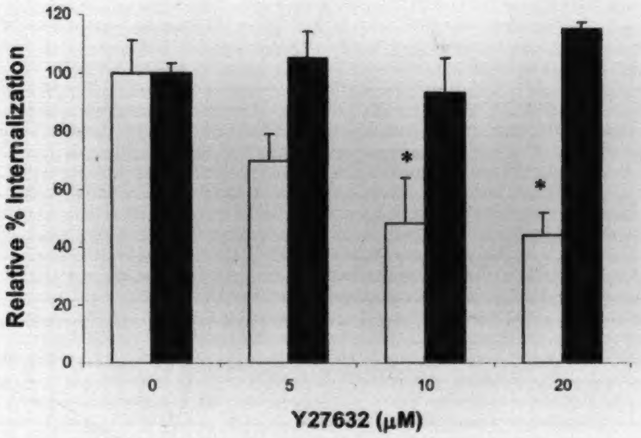

Fig. 5.6. Inhibition of ROCK, a downstream Rho effector, inhibits GBS internalization. Y27632, a ROCK inhibitor, was used to treat HeLa cells for 1 h prior to infection and inhibits GBS internalization but not that of *S. typhimurium*. ■ *S. typhimurium*; □ GBS. Relative percent internalization was calculated as follows: [(Number of CFU invaded into cells with inhibitor treatment/Number of CFU invaded into control cells) X 100%]. Error bars represent standard error of the mean (SEM). * $P < 0.05$ compared to control cells.

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Chapter 6:

Invasion of HeLa Cells by Group B *Streptococcus* Requires the Phosphoinositide-3-Kinase Signaling Pathway and Modulates Phosphorylation of Host-Cell Akt and Glycogen Synthase Kinase-3

A version of this chapter has been submitted for publication to the journal *Microbiology*.

6.1. Introduction

The Group B *Streptococcus* (GBS) is a Gram-positive, opportunistic bacterial pathogen with the ability to cause invasive disease in humans. GBS is best known for its ability to cause severe morbidity and mortality in neonates, and is the leading cause of meningitis in infants less than one month of age (Schuchat *et al.*, 1997;Schuchat, 1998;Tyrrell *et al.*, 2000). Other forms of invasive GBS neonatal disease include pneumonia and sepsis. GBS infections are not restricted to infancy, but can also occur in adulthood. Those at highest risk include the elderly and those with another underlying illness, such as diabetes mellitus (Farley *et al.*, 1993;Schuchat *et al.*, 1990;Schuchat, 1998;Tyrrell *et al.*, 2000). Disease manifestations in adulthood can be similar to those observed in neonates, in addition to bone and soft tissue infections.

It has been well established that GBS is able to invade a variety of host cell types, including epithelial cells, endothelial cells, and macrophages (Gibson *et al.*, 1993;Greco *et al.*, 1995;Lalonde *et al.*, 2000;Nizet *et al.*, 1997;Shin and Kim, 2006;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1996;Valentin-Weigand *et al.*, 1997). The attachment and invasion of GBS into host cells is thought to involve numerous pathogen-host cell interactions, but an understanding of these events at the molecular level is only in the early stages of characterization. What has been established is that host cell-actin microfilaments are recruited to the site of GBS attachment and invasion, and modulation of actin microfilaments by GBS is essential for invasion to occur. This has been demonstrated by the findings that the addition of cytochalasin D (a potent inhibitor of actin polymerization) to epithelial cells prior to infection inhibits GBS invasion (Gibson *et al.*, 1993;Greco *et al.*, 1995;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997), as well as microscopic demonstration of actin recruitment to the site of GBS attachment (Tyrrell *et al.*, 2002).

Phosphoinositide-3 Kinase (PI3K) is a lipid kinase that catalyzes the addition of a phosphate to phosphoinositides; the resulting phospholipid molecules modulate the actin cytoskeleton with precise spatial and temporal control (Stokoe, 2005;Vanhaesebroeck and Alessi, 2000). The formation of phosphatidylinositol 3,4,5-triphosphate (PIP_3) from phosphatidylinositol 3,4-bisphosphate (PIP_2) by PI3K leads to recruitment of Akt (Protein Kinase B) to the host cell membrane, and subsequent Akt phosphorylation and activation (for overview, see Fig. 6.1). Akt phosphorylation can subsequently trigger activation or inactivation of downstream Akt effectors, such as Glycogen Synthase Kinase-3 (GSK-3) (Cross *et al.*, 1995). The role of PI3K and phosphoinositide metabolism in bacterial pathogenesis is becoming increasingly appreciated. A requirement for PI3K in host cell invasion has been identified for the pathogens Group A *Streptococcus* (GAS) (Purushothaman *et al.*, 2003), *Helicobacter pylori* (Kwok *et al.*, 2002), *Chlamydia pneumoniae* (Coombes and Mahony, 2002), and *Listeria monocytogenes* (Ireton *et al.*, 1999), just to name a few. The result of manipulation of this pathway by a pathogen is coordination of actin rearrangement, leading to internalization of the organism.

The epithelium is a key component of innate immunity for humans. Breech of the epithelial barrier by a bacterial pathogen is often the precipitating event in invasive disease. In order to better understand the GBS disease process, my goal in this chapter was to explore the role of the PI3K/Akt signaling pathway in epithelial cell invasion by GBS.

6.2. Materials and Methods

6.2.1. Bacterial strains, cell lines, and growth conditions.

The GBS strain used in this investigation is NCS13, a serotype V organism that has been previously described (Tyrrell *et al.*, 2002). The *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain NCTC 10241 was obtained from the National Culture Collection, PHLS Colindale, London, England and the *Yersinia enterocolitica* subsp. *enterocolitica* (*Y. enterocolitica*) strain 23715 was obtained from the American Type Culture Collection (ATCC). All of the organisms were cultured at 35 °C on sheep blood agar plates (BAPs) (Dalynn Biologicals) or in Todd-Hewitt broth (THB) (Difco Laboratories).

The human epithelial cell line HeLa 229 (ATCC CCL-2.1) was obtained from the ATCC and was grown and maintained in OPTI-MEM I reduced serum medium (Invitrogen) supplemented with 4% fetal bovine serum (FBS) (Gibco), unless otherwise noted.

6.2.2. Chemical inhibitors.

Staurosporine, bisindolylmaleimide, manumycin A, and camptothecin (CPT) were purchased from Sigma-Aldrich. The PI3K inhibitor LY294002 was obtained from Cell Signaling Technology. The Akt Inhibitor ICIO (1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate), Akt Inhibitor V (Triciribine), and Akt Inhibitor VII were obtained from Calbiochem. Table 6.1 provides a summary of the actions of these inhibitors.

For the Akt inhibitor assays, HeLa cells were serum starved by replacing the OPTI-MEM I + 4% FBS with OPTI-MEM I (no FBS) for 18 to 24 h preceding 3 h inhibitor treatment prior to the addition of bacterial inoculum. For all other inhibitors, the HeLa cells were incubated for 1 h with inhibitor prior to adding bacterial inoculum. The inhibitors were present during the invasion assays until the first wash when fresh media was added, and during the entire duration of the attachment assays.

6.2.3. Antibodies and plasmids.

Rabbit anti-phospho-Akt (Ser 473) (9271) and rabbit anti-Akt (9272) were purchased from Cell Signaling Technology, as were mouse anti-phospho-GSK-3α (Ser21) (46H12; 9337), rabbit anti-phospho-GSK-3β (Ser9) (9336), and rabbit anti-GSK-3β (9315). Rabbit Caspase-3 antibody was obtained from Stressgen Bioreagents.

Plasmids expressing the dominant negative forms of the PI3K subunits (p110α; p85α) and the wild type p110α subunit (p110WT) were provided by Dr. David Stokoe (USCF Cancer Research Institute, San Francisco, CA) (Purushothaman *et al.*, 2003;Wang *et al.*, 2002).

6.2.4. Invasion (antibiotic protection) assay.

Invasion of bacteria into an epithelial cell (HeLa) monolayer was quantified using a standard antibiotic protection invasion assay with minor modifications (Rubens *et al.*, 1992;Tyrrell *et al.*, 2002). Briefly, a monolayer of HeLa cells was grown to confluence in 24 well plates (Corning) and then was treated with either a pharmacologic agent, or the solvent used as a carrier for the agent (solvent control; this is represented as the 0 μM concentration of inhibitor in the graphical representations of the data). Each assay condition was performed in triplicate. The bacterial inoculums (GBS, *S. typhimurium*, or *Y. enterocolitica*) were grown in THB at 35 °C with agitation overnight. The next day, approximately 1.5 X 10^5 GBS, 4 X 10^4 *S. typhimurium*, or 1.5 X 10^4 *Y. enterocolitica* were added to the monolayer. The difference in inoculum reflects the variation in invasion efficiency of the different species.

Following inoculation, the cell culture plates were centrifuged at 100 *g* for 5 min at room temperature. After a 2 h incubation at 35 °C to allow internalization, the monolayer was washed three times with phosphate buffered saline (PBS) to remove unbound bacteria. Bacteria that had bound but not internalized were killed by incubation for 2 h with fresh media containing 5 μg of penicillin per mL and 100 μg of gentamicin per ml. After the 2 h incubation, an aliquot of the media from each well was applied to a BAP to ensure that the antibiotic treatment was adequate to kill all extracellular bacteria. The monolayers were washed with PBS, trypsinized, and lysed with 0.1% Triton X-100. The lysates were spread onto BAPs and incubated overnight at 35 °C. The number of colonies on each plate were counted to determine the number of CFU that had invaded the monolayer.

The HeLa cell monolayers were examined at the end of each incubation period during the assay to observe their integrity in the presence of the chemical inhibitors. Portions of the HeLa cell culture supernatants were plated on to BAPs following the initial 2 h infection to ensure that the inhibitor treatments did not reduce bacterial viability. A subset of control invasion assays were also performed where the chemical inhibitor was added during the 2 h incubation with antibiotic-containing media rather than prior to adding the bacterial inoculum.

6.2.5. Transient transfection of HeLa cells.

For the invasion assays into HeLa cells with altered genetic backgrounds, a Lipofectamine transient transfection procedure with plasmid DNA expressing a dominant negative form of the cell moiety in question was utilized. HeLa cells were grown to approximately 80-90% confluence and the culture media was replaced with serum-free OPTI-MEM I. Then 1 μg of plasmid DNA and 2 μg of Lipofectamine™ 2000 Transfection Reagent (Invitrogen) were added to the HeLa cells in accordance with the manufacturer's instructions. After 5 h incubation at 37 °C, the cell culture media was replaced with fresh OPTI-MEM I with 4% FBS and the cells were incubated overnight at 37 °C prior to performing the invasion assay.

6.2.6. Bacterial attachment assay.

A quantitative attachment assay was performed to assess the ability of GBS to attach to a HeLa cell monolayer in the presence of the various pharmacologic inhibitors. HeLa cells were grown to confluence in 24 well tissue culture plates, then treated with inhibitor or the inhibitor's solvent, with each assay condition performed in triplicate. GBS were grown in THB at 35 °C with agitation overnight. The following day, approximately 3 X 10^4 GBS were added to the monolayer. For trypsinized GBS, a 0.5 McFarland standard of organism was made in 0.25% trypsin-PBS; this was incubated at 35 °C for 1 h and diluted in THB prior to inoculating HeLa cells.

Following inoculation, the cell culture plates were centrifuged at 100 *g* for 5 min at room temperature, and then incubated at 4 °C (a temperature that permits bacterial attachment but not internalization (Tamura *et al.*, 1994)) for 2 h. Following incubation, the HeLa cells were washed 6 times with PBS to remove unbound bacteria. The cells were then trypsinized and lysed with 0.1% Triton X-100. The lysates were spread onto BAPs and incubated overnight at 35 °C. The number of colonies on each plate were counted to determine the number of GBS that had attached to the monolayer under each assay condition.

6.2.7. Statistical analysis.

For the attachment and invasion assays, relative percent attachment/invasion was calculated as follows: [(Number of CFU attached (invaded) to treated cells/Number of CFU attached (invaded) to control cells) X 100%]. For each assay, every condition was performed in triplicate, and each assay was repeated a minimum of 3 times. GraphPad InStat3 software was used for statistical analysis of the data. Analysis of variance using Dunnet's Multiple Comparisons Test was used to compare attachment or invasion into inhibitor treated or transfected cells to that observed in the control cells. Where indicated, variance between inhibitor treatments was also analyzed with the Bonferroni Multiple Comparisons Test. A P value < 0.05 compared to control cells was deemed to be statistically significant and is denoted as "*" in the graphical representations of data. The graphical representations are illustrative of the mean of one representative experiment; error bars represent standard error of the mean (SEM).

6.2.8. Immunoblotting.

To assess modulation of Akt and GSK-3 phosphorylation in response to GBS infection, HeLa cells were grown in 6 well culture plates to approximately 80% confluence before being serum starved for 20 to 24 h. The cells were then challenged with an inoculum of 3 X 10^8 GBS for 5, 15, 30, or 60 min, or left uninfected (0 min). The reactions were immediately transferred to ice at the appropriate time points, the cells washed with cold PBS, and then harvested in ice-cold buffer containing 1X Cell Lysis Buffer (Cell Signaling Technologies), 0.2% Protease Inhibitor Cocktail for Mammalian Cell Extracts (Sigma-Aldrich), and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma-Aldrich). The lysates were centrifuged at 13 000 *g* for 10 min and the protein concentration of the supernatant fraction was determined using the Bio-Rad Protein Assay, based on the method of Bradford (Bradford, 1976). One hundred μg (for 10 well gels) or 50 μg (for 15 well gels) of cell lysate was resolved via SDS-PAGE, and electroblotted to a 0.45 μM nitrocellulose membrane (Bio-Rad) following standard protocols (Bollag and Edelstein, 1991). Each blot was then probed with antibody detecting a phosphorylated form of either Akt, GSK-3α, or GSK-3β, and detected on BioMax Light Film for Chemiluminescence (Kodak) using the Amersham Biosciences Enhanced Chemiluminescence (ECL) detection system. The blots were then stripped with Western Re-Probe Solution (Calbiochem), washed, and re-probed with either antibody detecting total Akt or total GSK-3β as loading controls. Where indicated, a subset of the cells was treated with 50 μM LY294002 or 20 μM ICIO prior to bacterial infection. For studies with heat-killed GBS (HK-GBS), a 3 McFarland standard of GBS was made in THB and then placed in an 80 °C water bath for 30 min. A portion of the culture was inoculated to a BAP to ensure the treatment was adequate for complete killing.

For immunoblots to detect Caspase-3 cleavage, HeLa cells were grown to approximately 90% confluence in 6 well culture dishes, serum starved for 18 to 20 h, and then infected with 2 X 10^8 CFU of log-phase growth GBS. Following a 30 min incubation to allow bacterial internalization to occur, the cells were washed and the media replaced with fresh media containing 5 μg of penicillin per mL and 100 μg of gentamicin per mL. At this time CPT (at the concentrations indicated in the assays) was also added to the cells for the incubation times indicated. Cell lysates were then collected, resolved, and probed as described for the phosphorylation studies with the exception that the primary antibody used was directed against Caspase-3.

6.3. Results

6.3.1. GBS invasion requires activation of PI3K.

To delineate host cell signaling events involved in GBS invasion of HeLa cells, the role of the PI3K signaling pathway in GBS invasion was explored. PI3K regulates numerous moieties related to cytoskeletal rearrangement, which is known to be a key event in GBS invasion of host cells. Wortmannin is a fungal metabolite that inhibits PI3K, mitogen-activated protein kinase and myosin light-chain kinase (Davies *et al.*, 2000). It has been previously illustrated that Wortmannin is a potent inhibitor of GBS epithelial cell internalization, suggesting that PI3K could be required in the invasion process (Tyrrell *et al.*, 2002). However, as Wortmannin can inhibit other host-cell kinases, confirmation of the specificity of this previous finding was sought using LY294002, a pharmacologic inhibitor that is specific for PI3K (Vlahos *et al.*, 1994). LY294002 inhibits PI3K by competing with ATP for binding to and activating PI3K, a different mechanism than that imparted by Wortmannin. Further, LY294002 has been demonstrated to inhibit GBS invasion into a different class of host cell, human brain microvascular endothelial cells (Shin *et al.*, 2006).

LY294002 reduced GBS invasion into HeLa cells at a concentration of 10 μM, and at 30 μM invasion was inhibited by approximately 75%. (Fig. 6.2a). In contrast, the internalization of *S. typhimurium*, a highly invasive bacterium that is internalized independently of PI3K (Finlay and Cossart, 1997;Tafazoli *et al.*, 2003), was not reduced in response to the LY294002 treatment.

An assay was also performed where LY294002 was added when the cell culture media was replaced with antibiotic-containing media following the 2 h bacterial infection period. This was to ensure that the inhibitor treatment was indeed reducing invasion of GBS rather than causing a decrease in intracellular viability of the organism. No reduction in GBS or *S. typhimurium* invasion was observed when LY294002 was added during the antibiotic treatment (Fig. 6.2b).

PI3K is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit. Transient transfection with a dominant negative form of the PI3K subunits was used to genetically inactivate PI3K and corroborate the findings of the chemical inhibitor studies. Invasion of GBS into HeLa cells expressing a dominant negative form of the p110α and the p85α subunits of PI3K was reduced by approximately 30% and 55%, respectively, compared to cells exposed to just the lipofectamine transfection reagent (Fig. 6.2d). While these figures are statistically significant, and the trend is in concurrence with the chemical inhibitor studies, the level of reduction in invasion is not as great as that observed with the chemical inhibitor treatment. This is likely due to the fact that this was a transient transfection assay and thus only a subset of the epithelial cell monolayer will have taken up and expressed the dominant negative form, and consequently posses inactive PI3K. HeLa cell invasion by *S. typhimurium* was not reduced by the genetic inactivation of either the p85α or the p110α subunit (Fig. 6.2d). As a further control, HeLa cells were transiently transfected with a wild-type p110α construct, which did not reduce GBS invasion into HeLa cells (Fig. 6.2d). These results, together with the previously reported Wortmannin data (Tyrrell *et al.*, 2002), illustrate that PI3K activation is required for the GBS invasion process.

In order to examine if PI3K is required for the adhesion of GBS to the host cell, a quantitative attachment assay was performed. Treatment with LY294002 did not reduce GBS attachment to HeLa cells at any concentration assayed (Fig. 6.2c). In each of the attachment assays presented in this study, trypsinized GBS were employed as a control for detection of reduced attachment, as it has been previously demonstrated that while trypsinization does not reduce GBS viability, it cleaves surface proteins important in adherence to epithelial cells (Tamura *et al.*, 1994), thus qualifying that this assay system would detect an alteration in GBS attachment to host cells should one be present.

6.3.2. Entry of GBS into epithelial cells requires Protein Kinase activity.

PI3K activation results in the production of phosphorylated lipid products in the cell membrane, which can lead to the recruitment of other host cell proteins (Takenawa and Itoh, 2001), and activation of downstream signaling moieties. One class of effectors influenced by PI3K are protein kinases, and thus the involvement of these in GBS invasion was investigated as well. I began with staurosporine, a broad-spectrum protein kinase inhibitor. Previous studies suggested that this inhibitor reduces but does not abolish GBS invasion into HEp-2 cells (Valentin-Weigand *et al.*, 1997). It was found that staurosporine completely abolished GBS invasion of HeLa cells, even at the very low concentration of 0.2 μM, but failed to inhibit *S. typhimurium* invasion, even at 1.0 μM, suggesting that protein kinases are involved in the GBS internalization pathway (data not shown). Protein Kinase C (PKC) comprises a major proportion of the protein kinase family and is a downstream target of PI3K (Chou *et al.*, 1998). Further, PKC has been shown to interact with host cell proteins involved in the anchoring of actin microfilaments in the host cell, and thus seemed a likely protein kinase candidate. Bisindolylmaleimide, a PKC inhibitor, was assayed for an effect on GBS invasion of HeLa cells. Bisindolylmaleimide did not inhibit GBS invasion at the maximal concentration assayed (1.0 μM) and *S. typhimurium* was also unaffected by the treatment (Fig. 6.3a). Another control organism, *Y. enterocolitica*, was also assayed to ensure that the bisindolylmaleimide was having the expected effects on the host cell at the concentrations used. The invasion of *Y. enterocoltica* was reduced over all the concentrations assayed, from 0.1 to 1.0 μM (Fig. 6.3a). This data suggested that PKC is not involved in GBS invasion, and that other protein kinase or protein kinase pathways must be utilized; thus, an alternate protein kinase, Akt (Protein Kinase B) was considered.

6.3.3. Akt is required for HeLa cell invasion by GBS.

The Akt inhibitor 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (ICIO) is a phosphatidylinositol ether analogue that inhibits Akt by preventing PIP_3 from forming and binding to Akt (Fig. 6.1, step 2) (Hu *et al.*, 2000). This inhibitor is reported to be selective and specific for Akt up to the concentration of 83 μM, and was utilized to screen for the potential involvement of this protein kinase in GBS infection. The ICIO treatment resulted in a dose-dependent inhibition of GBS that became statistically significant at 20 μM, with a 50% reduction in invasion, suggesting Akt is involved in GBS invasion (Fig. 6.3b). A quantitative attachment assay indicated that ICIO did not reduce GBS adhesion to the HeLa cells at any concentration assayed (Fig. 6.3d).

As illustrated in Fig. 6.1, activation of Akt occurs in a series of sequential steps involving binding to PIP_3 followed by two sequential phosphorylation events. Akt inhibitors are available that function at different levels of Akt activation; I wanted to investigate if different modes of Akt inhibition would affect GBS invasion differently. To this end, Akt Inhibitor V (Akt V) and Akt Inhibitor VII (Akt VII) were used in conjunction with the invasion assay. Akt V inactivates Akt by targeting a process that is yet to be clearly characterized, but is known not to involve PI3K or phosphoinositide-dependent protein kinase 1 (PDK1) (i.e. it blocks a process other than 1 or 4 in Fig. 6.1) (Yang *et al.*, 2004). Akt VII is a peptide that has been engineered to permeate the host cell and inactivates Akt by binding to the plesktrin homology (PH) domain of Akt, thereby preventing phosphoinsoitide binding by Akt (step 3, Fig. 6.1) (Hiromura *et al.*, 2004). Each of the Akt inhibitors reduced GBS invasion in a significant fashion, but the level of invasion inhibition between these three inhibitors did not differ significantly (Fig. 6.3e).

Invasion assays were also performed where the Akt inhibitors were added after internalization, during the antibiotic treatment. These assays indicated that none of the Akt inhibitors, at the concentrations used, reduced bacterial viability inside the HeLa cells (Fig. 6.3c and 3f).

6.3.4. Ras is involved in GBS invasion of HeLa Cells.

It has been demonstrated that Ras can activate PI3K by targeting the p85 domain, which leads to activation of the PI3K/Akt pathway (Chan *et al.*, 2002). In light of the finding that PI3K and Akt are required for GBS invasion, the pharmacologic inhibitor Manumycin A was used to investigate the role of Ras in GBS internalization. This compound is a Ras farnesyltransferase inhibitor that functions by blocking post-translational isoprenylation of Ras required for proper membrane binding and targeting, which is essential for Ras activation (Sattler *et al.*, 1998).

The treatment of HeLa cells with Manumycin A for 1 h prior to GBS infection had a potent, dose-dependant inhibitory effect on invasion. At a concentration of 2.5 μM, 30% inhibition of invasion occurred (Fig. 6.4a) and at 10 μM, invasion was reduced by 90%. In contrast, the invasion of *S. typhimurium* was not affected by the treatment (Fig. 6.4a). When Manumycin A was added during the incubation with antibiotic-containing media, a slight, statistically insignificant reduction in invasion was observed at 10 μM Manumycin A (Fig. 6.4b). Manumycin A did not alter the ability of GBS to attach to HeLa cells in a significant manner (Fig. 6.4c).

These results suggest that Ras is also involved in the signaling pathway leading to the internalization of GBS into epithelial cells.

6.3.5. Phosphorylation of Akt in HeLa cells occurs in response to GBS infection.

Akt is a serine/threonine kinase that is phosphorylated in its active form. Although a need for active Akt was demonstrated in the chemical inhibitor studies, it was not clear if GBS was able to modulate Akt phosphorylation directly. To assay for Akt phosphorylation during GBS invasion, HeLa cells were either infected with GBS or left uninfected, then lysates were collected and assayed via Western blot using an antibody recognizing the activated form of Akt that is phosphorylated on Ser 473. The blot was then stripped and reprobed for total Akt as a loading control, confirming that the observed effects are due to modulation of Akt phosphorylation status and not an increase in protein synthesis in the cell.

Akt phosphorylation in HeLa cells was induced by GBS infection at the early time point of 5 min and continued for at least 60 min post infection (Fig. 6.5a). Akt phosphorylation was not observed in HeLa cells infected with heat-killed GBS (HK-GBS) (Fig. 6.5d).

The demonstration of PI3K and Akt involvement in GBS internalization suggested transduction through PI3K as an upstream modulator of Akt phosphorylation. To explore this, a subset of cells were treated with 50 μM LY294002 for 1 h prior to GBS infection. This treatment abolished Akt phosphorylation (Fig. 6.5a), illustrating that GBS induced Akt phosphorylation occurs downstream from PI3K activation.

6.3.6. GBS infection results in phosphorylation of Glycogen Synthase Kinase-3 (GSK-3).

Akt has been demonstrated to play a role in the balance between host cell survival and cell death as a mediator of apoptosis. Akt phosphorylates many targets that modulate apoptotic function in the host cell, with one major target being GSK-3. GSK-3 exists in two isoforms, GSK-3α (51 kDa) and GSK-3β (47 kDa), and is primarily regulated through inhibition. Phosphorylation of GSK-3 is inhibitory, and is a pro-survival signal in the host cell. To explore modulation of host-cell GSK-3 phosphorylation by GBS, HeLa cells were either left uninfected, or infected with GBS for 5, 15, 30, or 60 min, then cell lysates were collected and analyzed using Western blot with antibodies recognizing the phosphorylated form of GSK-3α (Ser21), or GSK-3β (Ser9). The blots were then stripped and re-probed with an antibody recognizing total GSK-3β as a control on protein loading.

GBS infection results in an increase in GSK-3α phosphorylation at 5 min post infection and this continues for at least 60 min post infection (Fig. 6.5b). GBS infection also stimulates GSK-3β phosphorylation, but the effect is more subtle and does not occur until 15 min post infection (Fig. 6.5b). HK-GBS do not induce GSK-3 phosphorylation (Fig. 6.5d). GBS induced phosphorylation of GSK-3 α and β is abolished by treatment with LY294002 for 1 h prior to GBS infection (Fig. 6.5b). However, PI3K does have downstream effectors other than Akt so a subset of cells were also treated with ICIO for 3 h prior to GBS infection. Inhibition of Akt prevents GBS induced GSK-3 phosphorylation until 30 min post infection, but at 60 min, some phosphorylation is observed (Fig. 6.5c). However, at this point, some phosphorylation of Akt is also observed (Fig. 6.5c), suggesting that after that amount of time, GBS infection of the cells overwhelms the ability of the inhibitor to completely eliminate levels of phosphorylated Akt in the HeLa cell. This data indicates that GSK-3 phosphorylation induced by GBS occurs downstream of PI3K and Akt.

6.3.7. Infection with GBS protects HeLa cells from camptothecin (CPT)-induced Caspase-3 cleavage.

CPT is a topoisomerase inhibitor that induces apoptosis transduced by Caspase-3 cleavage. As the phosphorylation of Akt and GSK-3 are well recognized pro-survival (anti-apoptotic) signals, the possibility that GBS infection could protect HeLa cells from the activity of CPT was considered. HeLa cells were either infected with GBS for 30 min, or left uninfected. The cell media was then replaced with fresh media containing antibiotics and either 0, 10, or 50 μM CPT for 4 h, at which time HeLa cell lysates were collected and analyzed by Western blot. Caspase-3 was detected using an antibody that recognizes the uncleaved, procaspase form (approximately 35 kDa) as well as the 20 kDa processed (activated) form. In the uninfected cells, Caspase-3 cleavage was observed in both the 10 and 50 μM CPT treatments (Fig. 6.6a). In contrast, Caspase-3 cleavage was not observed in the infected cells at either CPT concentration (Fig. 6.6a). The assay was then extended to examine the ability of GBS to protect HeLa cells from Capase-3 cleavage at longer time points post infection, and to observe if the balance might shift to become pro-apoptotic at later time points. At 18 h post infection, low levels of Caspase-3 cleavage are observed in CPT treated, GBS infected cells, but the amount is much lower than that observed in uninfected, CPT treated cells (Fig. 6.6b). The assay could not be performed at later time points, because at approximately 20 h post infection, some Caspase-3 cleavage began to appear in the untreated control cells, likely owing to the advanced age and prolonged period of serum starvation of the cells by this point in time (not shown). These data suggest that GBS promotes host cell survival at early points post-infection, and can prevent or delay activation of Caspase-3, the executioner caspase of apoptosis.

6.4. Discussion

The PI3K/Akt signaling pathway has been implicated in an array of cellular functions, including cytoskeletal regulation, vesicle trafficking, and the balance between cellular survival or regulated cell death (Stokoe, 2005). PI3K activation specifically is implicated in phagocytosis, pseudopod formation, and membrane ruffling; the formation of these cellular structures is dependant upon modification and manipulation of the actin cytoskeleton, a key event in GBS invasion of host cells (Cox *et al.*, 1999;Pizarro-Cerda and Cossart, 2004;Stokoe, 2005;Tyrrell *et al.*, 2002). In this chapter, HeLa cells were used as a model of the host epithelial cell barrier. LY294002 treatment of HeLa cells inhibited GBS internalization, confirming previous findings suggesting that PI3K is involved in the GBS epithelial cell invasion process (Tyrrell *et al.*, 2002), as well as in GBS invasion into brain microvascular endothelial cells (Shin *et al.*, 2006).

PI3K activation triggers activation of a number of downstream signaling molecules, including protein kinases. It was found that while protein kinases play a role in GBS invasion into HeLa cells, PKC appears not to be involved. Purushothaman *et al.* (Purushothaman *et al.*, 2003) found this to be the case for invasion of GAS into HEp-2 cells as well. However, for *Streptococcus suis*, it was found that PKC inhibitors reduce phagocytosis into J774 macrophage cells (Segura *et al.*, 2004). *S. suis* infection of these cells resulted in rapid phosphorylation of PKC post infection, and this occurred downstream from PI3K activation (Segura *et al.*, 2004). Thus, while I have demonstrated that inhibition of PKC does not affect GBS internalization into HeLa cells, it is possible that the scenario is different in other cell types.

Ras is a GTP-binding switch protein that cycles between the active, GTP bound state and the inactive, GDP bound state (Reuther and Der, 2000;Taylor and Shalloway, 1996). Ras signaling is initiated by ligand binding to different receptors at the host cell surface, and functions downstream from receptor kinases (Reuther and Der, 2000). Ras itself also activates downstream effector molecules; PI3K can be recruited to the host cell membrane for activation via activated Ras (Reuther and Der, 2000). In light of identifying a requirement for PI3K in GBS invasion, it was not surprising that Manumycin A, a Ras inhibitor, also inhibited GBS internalization.

One category of PI3K effectors is the Rho family GTPases; these GTPases are also implicated in many aspects of actin regulation. For example, Rac, Rho, and Cdc42 link extracellular signals to the formation of stress fibers, lamellipodia, or filopodia (Hall, 1998;Tapon and Hall, 1997). Rho-family GTPases are important in GBS invasion into HeLa cells (Chapter 5) as well as human brain microvascular endothelial cells (HBMEC) (Shin and Kim, 2006), and thus it is possible that interplay between PI3K and Rho GTPases mediates GBS invasion as well. However, the PI3K-Rho GTPase relationship can be complex; these molecules can act both upstream and downstream from one another, and thus further investigation is required to solidify the interaction between these signaling pathways in GBS invasion.

Akt inhibitor assays illustrated that the PI3K effector Akt is required for efficient GBS invasion of HeLa cells. The suggestion that Akt is involved in GBS invasion is strengthened by the observation that Akt is phosphorylated in response to GBS infection; Akt phosphorylation occurs quickly after GBS is added to HeLa cells and persists for at least 60 min post infection. The ability of Akt to regulate so many facets of the host cell makes it an attractive target for a bacterial pathogen to manipulate to augment the invasion process.

In addition to cytoskeletal regulation, Akt plays a role in the balance between host cell survival and cell death as a mediator of apoptosis. Akt phosphorylates several targets that modulate apoptotic function (Chan *et al.*, 1999;Datta *et al.*, 1999). Thus, it was hypothesized that Akt activation by GBS may be a mechanism to contribute to bacterial persistence inside the host cell by inhibiting apoptotic events. This has recently been demonstrated to occur in the infection of epithelial cells by *S. typhimurium* (Knodler *et al.*, 2005), in which the SopB protein of *S. typhimurium* activates Akt and prevents Caspase-3 mediated apoptosis (Knodler *et al.*, 2005). Thus, it was a natural progression to assay for modulation of GSK-3 phosphorylation in response to GBS infection. GSK-3 is a multifunctional serine/threonine kinase that acts downstream from Akt in the PI3K/Akt signaling pathway (Embi *et al.*, 1980). GSK-3 was the first target of Akt to be identified (Cross *et al.*, 1995), and while GSK-3 was first acknowledged for its role in glycogen metabolism, subsequently it has been found to behave as a somewhat promiscuous molecular switch that is activated by diverse stimuli and regulates numerous cellular processes and pathways, including regulation of several transcription factors.

There are two GSK-3 isoforms (GSK-3α, 51 kDa and GSK-3β, 47 kDa) which are ubiquitously expressed and highly conserved in mammalian cells, although they are encoded by distinct genes. GSK-3 is somewhat unusual as it is regulated mainly by inhibition, rather than activation. It has been demonstrated that PI3K activation of Akt results in phosphorylation of both GSK-3α and GSK-3β (Cross *et al.*, 1995); this phosphorylation inhibits GSK-3 activity, leading to dephosphorylation of GSK-3 substrates.

A role for GSK-3 in host cell survival was identified in 1998 by Pap and Cooper (Pap and Cooper, 1998), who found that overexpression of active GSK-3 induced apoptosis, and that inhibition of GSK-3 (mediated by a dominant negative) prevented apoptosis downstream from PI3K inhibition. In this chapter, it was observed that GBS infection of HeLa cells results in phosphorylation (inactivation) of both the α and β isoforms of GSK-3. This phosphorylation occurs quickly after infection with GBS, and persists for at least 60 min post infection. The induction of pro-survival signals in the host epithelial cell lead me to wonder if infection by GBS would be sufficient to protect HeLa cells from apoptosis induced by the pharmacologic agent camptothecin (CPT). I found that GBS infection protects

HeLa cells from CPT-induced apoptosis, and that this effect lasts for at least 18 h post-infection.

Modulation of the balance between host-cell survival and host-cell death is a popular strategy for survival utilized by bacterial pathogens. One example is induction of apoptosis in phagocytic cells, such as the macrophage, to avoid bacterial killing. Another approach is to avoid or delay apoptosis to seek refuge from the immune system of the host, or gain access to privileged sites. Regardless of the purpose, the fact that a wide range of intracellular organisms are able to modulate host cell apoptosis illustrates that this function is highly conserved through evolution, suggesting it is deliberate and likely important in the pathogenic process.

Previous investigations into GBS invasion found that GBS were able to persist inside A549 and HUVEC cells for 8 h post infection, and while they did not replicate, the number of intracellular GBS did not decline (Gibson *et al.*, 1993;Rubens *et al.*, 1992). Similar results were found in later studies investigating the ability of GAS and GBS to invade cultured cells. Greco and colleagues (Greco *et al.*, 1995) observed that GBS remain viable in HeLa, HEp-2, and HUVEC cells for 24 h after infection, and again observed that while the GBS do not replicate, they also do not decrease in number. In contrast, there was a gradual decrease in intracellular GAS over the 24 h period, with no viable intracellular GAS found at 24 h post-infection in any of the 3 cell lines assayed (Greco *et al.*, 1995). Nizet *et al.* observed that GBS are also able to persist inside brain microvascular endothelial cells for at least 20 h after infection without replication (Nizet *et al.*, 1997). The fact that GBS are able to induce pro-survival signals inside the HeLa cell suggests a possible mechanism for GBS persistence in epithelial cells for relatively long periods of time.

Ulett *et al.* have demonstrated that GBS activates caspase-3 and –9 in macrophages at 48 h post infection, leading to apoptosis (Ulett *et al.*, 2005). It is possible that while GBS protects HeLa cells from apoptosis at early time points (up to 24 h) post infection, that after longer time points have past, apoptosis becomes activated. This is a potential topic for future investigation.

Although in some situations GBS causes asymptomatic colonization of the host, the ability of GBS to invade epithelial cells is central to the ability of the organism to cause invasive disease, a process contributing to the clinical features of GBS infection. Research targeted at elucidation of the interface between GBS and the host is an important avenue towards the development of novel therapeutic agents. In theory, an organism should be less likely (or slower) to develop resistance to therapies targeted to bacterial pathogenicity rather than survival, and such therapies should be less disruptive to the normal flora of the body. However, the interaction between GBS and host epithelial cells is complex, and several facets are still unclear, including identification of the events occuring at the cell surface that trigger PI3K activation and the subsequent signaling cascade.

In summary, it is well established that GBS invade non-phagocytic host cells; the objective in the present chapter was to characterize the GBS epithelial cell invasion process. HeLa cells were used as the infection model to explore signal transduction processes involved between GBS and epithelial cells during invasion. The results from this investigation illustrate that GBS both requires and activates the PI3K/Akt host cell signaling pathway during invasion.

Table 6.1. Pharmacologic agents used in this investigation.

Agent	Target or Mode of Action in Host cell
Akt Inhibitor ICIO	Inhibits Akt by preventing PIP_3 formation
Akt Inhibitor V	Inhibits Akt; mechanism yet to be characterized but does not involve PI3K or PDK1
Akt Inhibitor VII	Interacts with the PH domain of Akt to prevent phosphoinositide binding by Akt
Bisindolylmaleimide	Interacts with PKC catalytic subunit to competitively inhibit ATP binding
Camptothecin	Topoisomerase inhibitor; induces Caspase-3 cleavage
LY294002	PI3K inhibitor; competes with ATP for binding to and activation of PI3K
Manumycin A	Ras farnesyltransferase inhibitor; blocks membrane binding that is essential for Ras activation
Staurosporine	Broad-spectrum protein kinase inhibitor

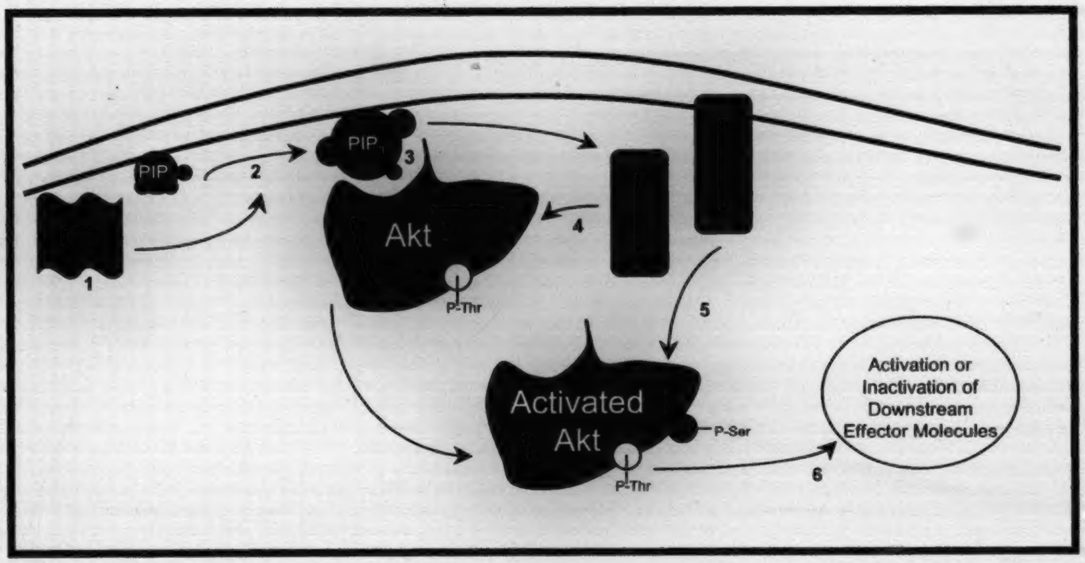
PIP
2
PIP
3
1
Akt
P-Thr
4
5
Activated
Akt
P-Ser
P-Thr
6
Activation or
Inactivation of
Downstream
Effector Molecules

Fig. 6.1. Schematic diagram illustrating events in Akt activation by the PI3K pathway. An external stimulus binds to a receptor on the host cell surface, leading to activation of PI3K (1), catalyzing the formation of membrane-bound PIP_3 from PIP_2 (2). Akt is then recruited to the cell membrane, where it is anchored via binding of PIP_3 to the PH domain of Akt (3). This is followed by phosphorylation of a threonine residue (Thr^{308}) on the Akt kinase domain by Phosphoinositide-dependent kinase-1 (PDK1) (4) resulting in a conformational change in Akt and phosphorylation of a serine residue (Ser^{473}) by PDK2 (5), leading to full activation of Akt kinase activity. Akt can then interact with various effector molecules in the host cell (6), resulting in activation or inactivation of these host cell components.

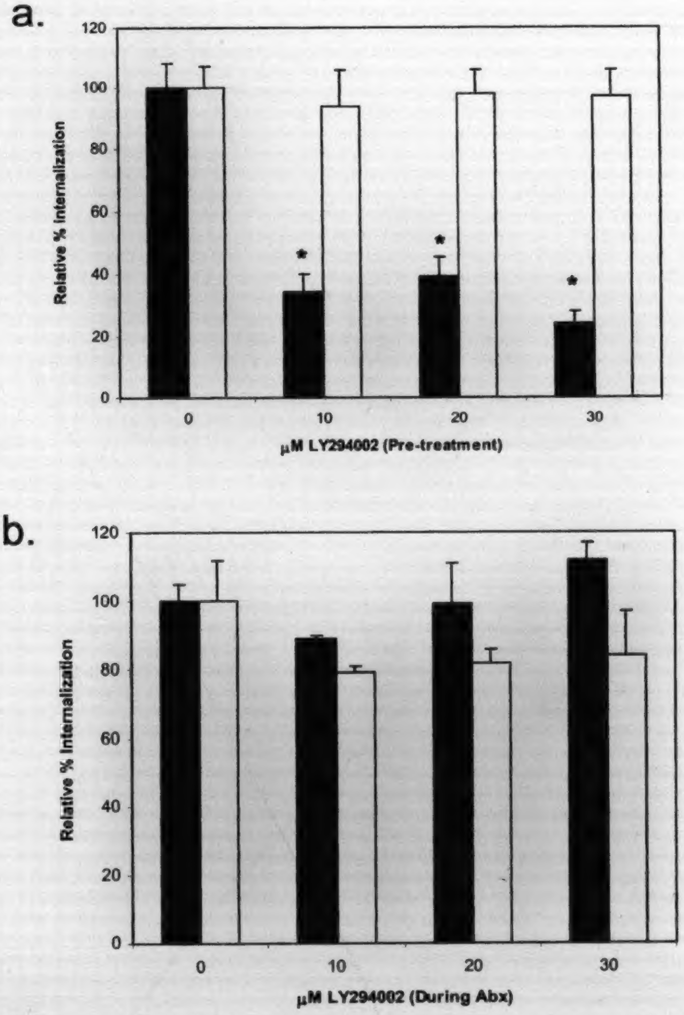

Fig. 6.2. HeLa cell invasion (but not attachment) by GBS requires PI3K. Solid bars (■) GBS; open bars (□) *S. typhimurium*. (a). The HeLa cell monolayer was treated with the PI3K inhibitor LY294002 for 1 h prior to bacterial infection; this treatment inhibited GBS invasion but not that of *S. typhimurium*. (b). If LY294002 was added during HeLa cell treatment with antibiotic containing media, no reduction in GBS or *S. typhimurium* invasion was observed, indicating this inhibitor does not reduce intracellular viability of these organisms. (Continued next page).

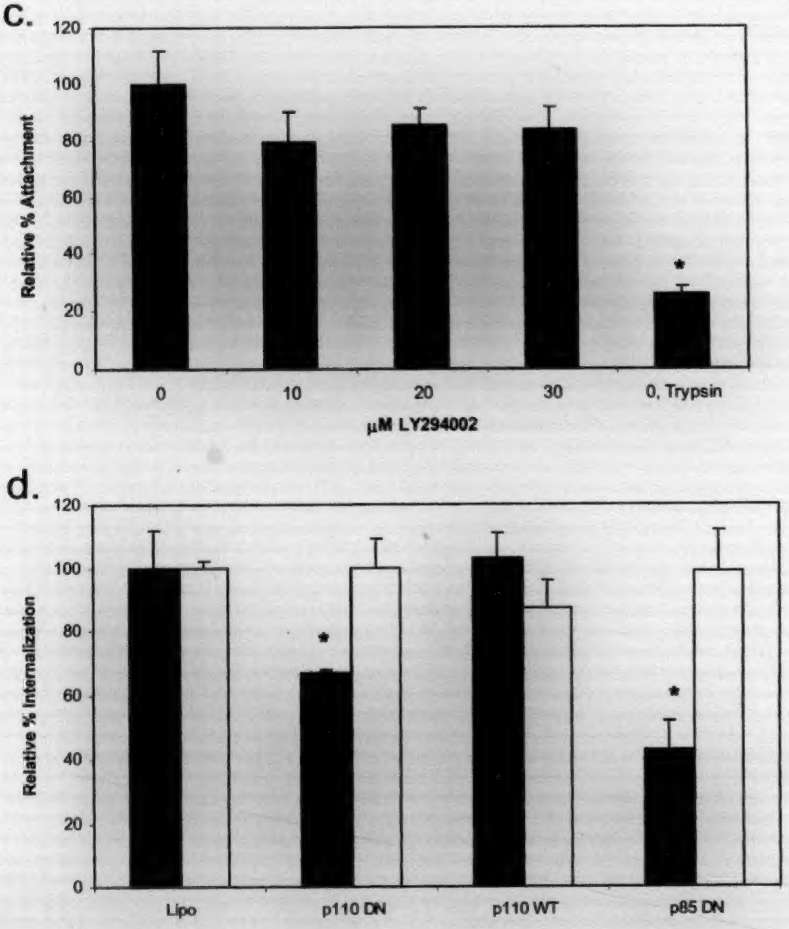

Fig. 6.2 (continued). (c). Treatment of HeLa cells with LY294002 for 1 h prior to GBS infection does not reduce bacterial attachment to these cell lines; attachment of GBS to HeLa cells is impaired if the bacteria are treated with a 0.25% trypsin solution for 1 h at 35 °C prior to infection. (d). Transfection of HeLa cells with dominant negative PI3K catalytic (p110α) or regulatory (p85α) subunits inhibits GBS invasion but not *S. typhimurium* invasion. In contrast, transfection of the cells with wild type p110α does not inhibit the ability of GBS to invade the cells. Relative percent attachment/internalization was calculated as follows: [(Number of CFU invaded (attached) into treated cells/Number of CFU invaded (attached) into control cells) X 100%]. Graphical representations are illustrative of the mean one representative experiment in which each assay condition was performed in triplicate; each assay was performed at least 3 times. Error bars represent standard error of the mean. * indicates a *P* value < 0.05 compared to control cells as evaluated using Dunnet's Multiple Comparisons Test.

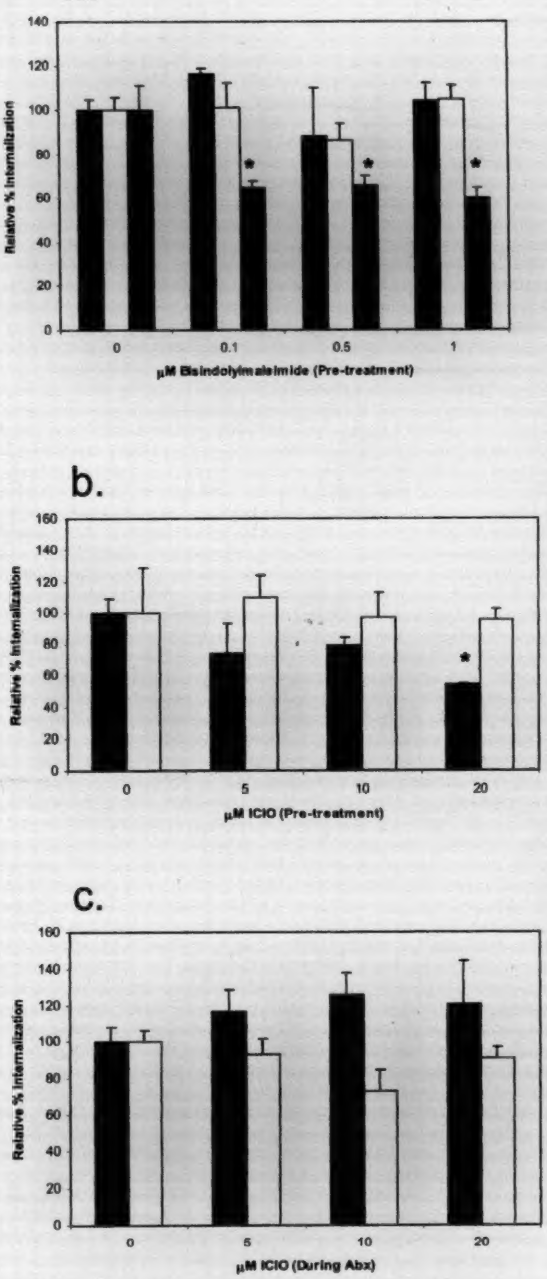

Fig 6.3. GBS invasion requires Akt but not PKC. Solid bars (■) GBS; open bars (☐) *S. typhimurium*; shaded bars (■) *Y. enterocolica.* (a). Bisindolylmaleimide, a PKC inhibitor, was used to treat HeLa cells for 1 h prior to bacterial infection. The treatment did not inhibit GBS or *S. typhimurium* invasion but did reduce the invasion of *Y. enterocolitica.* (b). HeLa cells were serum starved for 18 h and then treated with the Akt inhibitor ICIO for 3 h prior to infection. This treatment inhibited GBS invasion in a dose dependent manner that became significant at 20 µM. (d). GBS attachment is not inhibited by ICIO treatment. (e). HeLa cells were serum starved for 20 h and then treated with ICIO or the Akt inhibitors Akt V or Akt VII for 3 h prior to infection. Each of these treatments inhibited GBS invasion, however the level of inhibition induced by the different agents did not differ significantly ($P \geq 0.05$ with Bonferroni Multiple Comparisions analysis of variance comparing pairs of columns). (Continued next page).

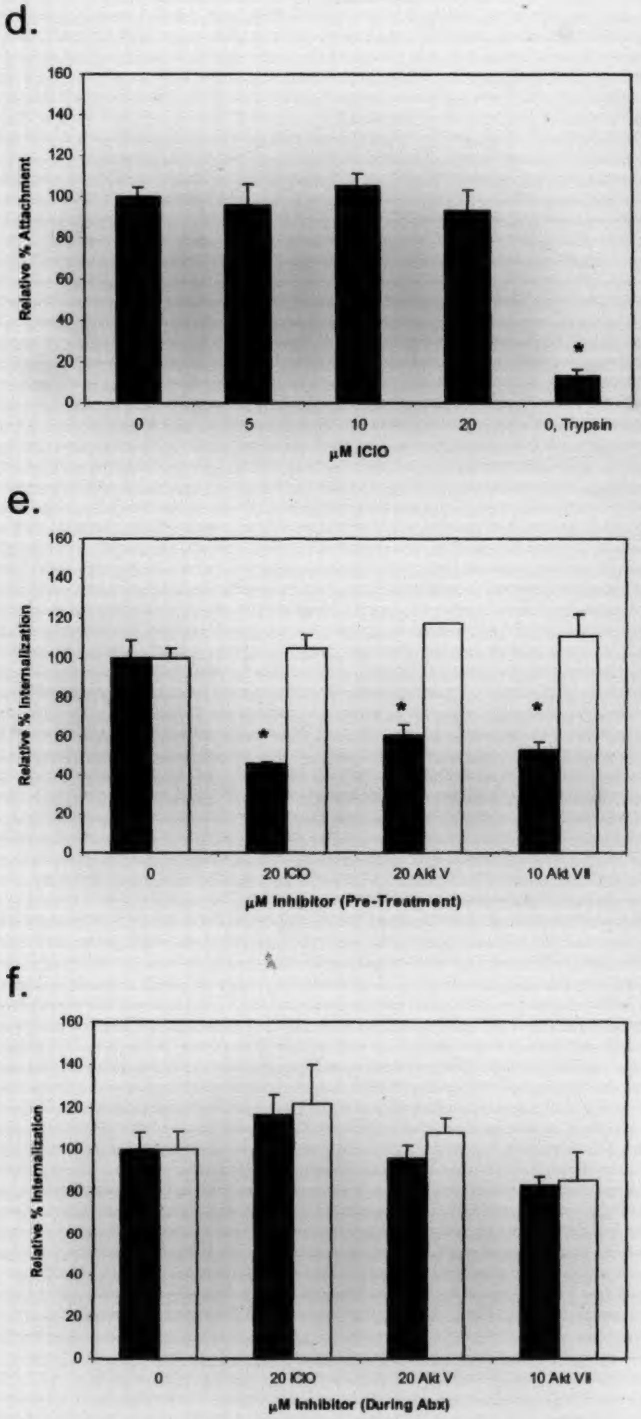

Fig. 6.3 (continued). If the Akt inhibitors ICIO (c, f), Akt V (f) or Akt VII (f) are added during HeLa cell treatment with antibiotic containing media rather than prior to infection, no reduction in GBS invasion or intracellular viability is observed. Relative percent attachment/internalization was calculated as follows: [(Number of CFU invaded (attached) into treated cells/Number of CFU invaded (attached) into control cells) X 100%]. Graphical representations are illustrative of the mean one representative experiment in which each assay condition was performed in triplicate; each assay was performed at least 3 times. Error bars represent standard error of the mean. * indicates a *P* value < 0.05 compared to control cells as evaluated using Dunnet's Multiple Comparisons Test.

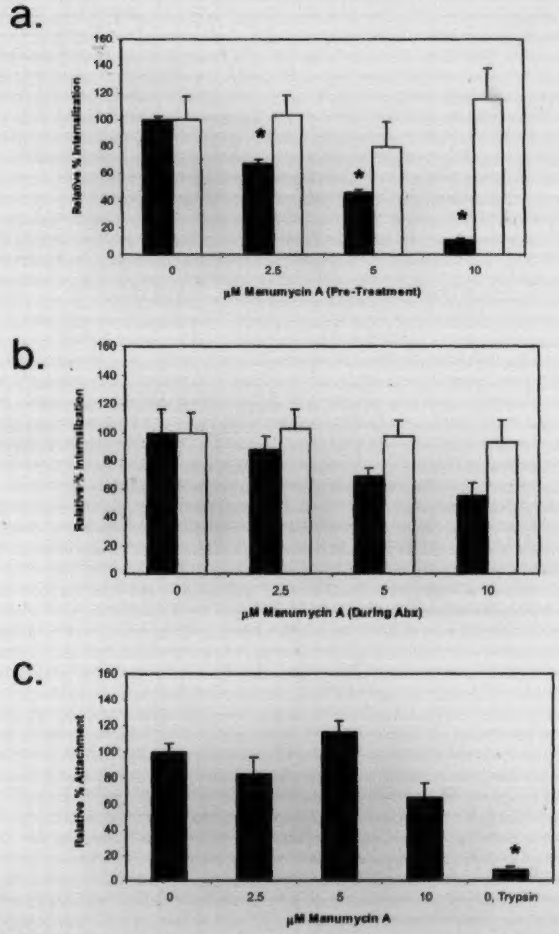

Fig. 6.4. Ras is required for GBS invasion into HeLa cells. Manumycin A, an inhibitor of Ras farnesyltransferase, was used to treat HeLa cells for 1 h prior to bacterial infection (a); this treatment inhibited GBS invasion in a dose dependant manner but did not inhibit *S. typhimurium* invasion; the treatment did not inhibit GBS attachment to HeLa cells (c). If Manumycin A was added during HeLa cell incubation with antibiotic containing media following bacterial invasion (b), no significant reduction in GBS intracellular viability was observed. Relative percent attachment/internalization was calculated as follows: [(Number of CFU invaded (attached) into treated cells/Number of CFU invaded (attached) into control cells) X 100%]. Graphical representations are illustrative of the mean one representative experiment in which each assay condition was performed in triplicate; each assay was performed at least 3 times. Error bars represent standard error of the mean. * indicates a *P* value < 0.05 compared to control cells as evaluated using Dunnet's Multiple Comparisons Test.

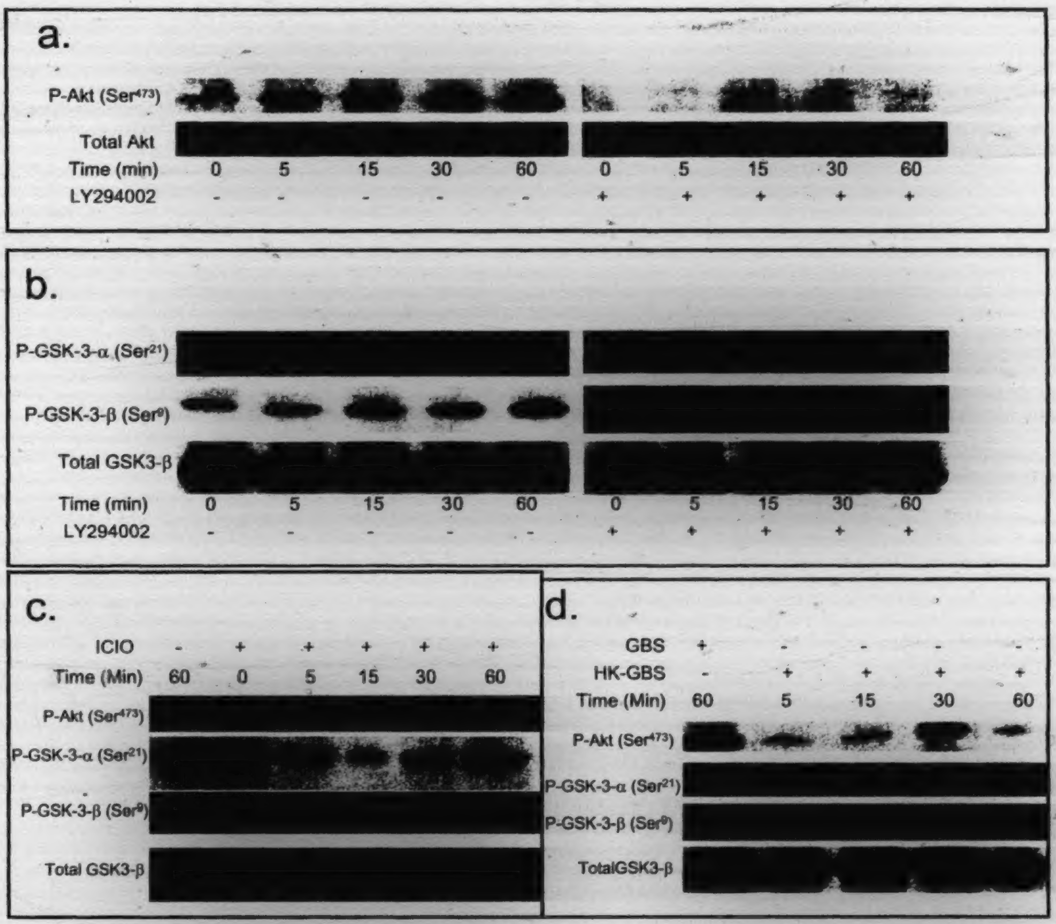
a.
P-Akt (Ser473)
Total Akt
Time (min) 0 5 15 30 60 0 5 15 30 60
LY294002 - - - - - + + + + +
b.
P-GSK-3-α (Ser21)
P-GSK-3-β (Ser9)
Total GSK3-β
Time (min) 0 5 15 30 60 0 5 15 30 60
LY294002 - - - - - + + + + +
c.
ICIO - + + + + +
Time (Min) 60 0 5 15 30 60
P-Akt (Ser473)
P-GSK-3-α (Ser21)
P-GSK-3-β (Ser9)
Total GSK3-β
d.
GBS + - - - -
HK-GBS - + + + +
Time (Min) 60 5 15 30 60
P-Akt (Ser473)
P-GSK-3-α (Ser21)
P-GSK-3-β (Ser9)
TotalGSK3-β

Fig. 6.5. GBS invasion of HeLa cells induces phosphorylation of Akt and GSK-3. (a). Immunoblot for Akt phosphorylation. Serum-starved HeLa cells were either left untreated (0 min) or treated with GBS for 5, 15, 30, or 60 min. Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. Phosphorylated Akt was detected using an anti-phospho-Akt (Ser^{473}) antibody followed by detection of total cellular Akt with a polyclonal antibody. A subset of cells were also treated with 50 μM LY294002 for 1 h prior to GBS infection. (b, c). Immunoblots for GSK-3 phosphorylation. Serum-starved HeLa cells were either left untreated (0 min) or infected with GBS for 5, 15, 30, or 60 min. Subsets of HeLa cells were treated with 50 μM LY294002 for 1 h or 20 μM Akt inhibitor ICIO for 3 h prior to GBS infection. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose, and probed with antibodies recognizing GSK-3α phosphorylated on serine 21 or GSK-3β phosphorylated on serine 9. Blots were then re-probed with antibody detecting total cellular GSK-3β. (d). Immunoblot for phosphorylation of Akt, GSK-3α, and GSK-3β in HeLa cells in response to infection with GBS or heat-killed GBS (HK-GBS).

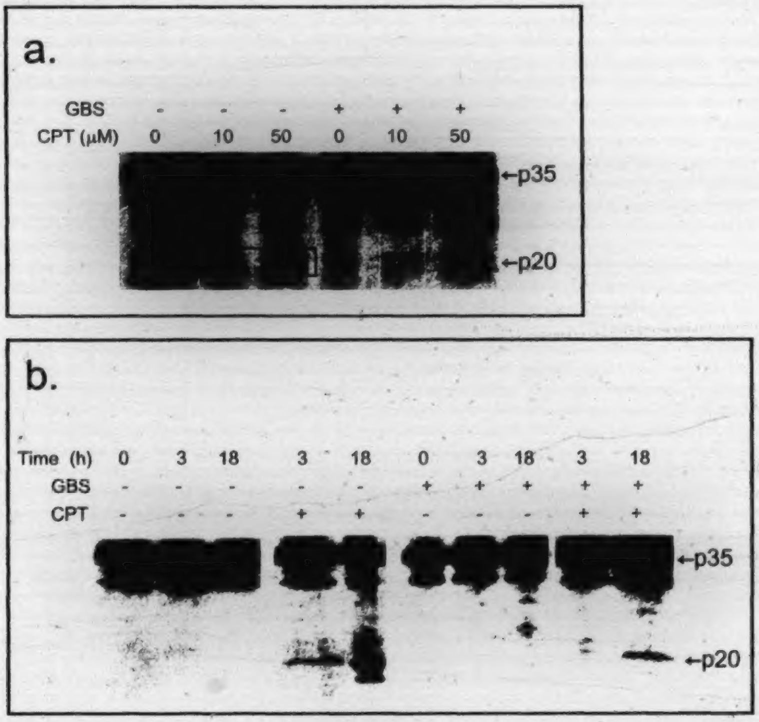

Fig. 6.6. GBS infection protects HeLa cells from CPT induced Caspase-3 cleavage. (a). HeLa cells were either infected with log-phase growth of GBS for 30 min or left uninfected. The cellular media was then replaced with fresh, antibiotic containing media and treated with 0, 10, or 50 μM CPT for 4 h, at which time the HeLa cells were collected, the lysastes separated by SDS-PAGE and transferred to nitrocellulose. Caspase-3 was detected using an antibody that detects both the uncleaved, procaspase form of Caspase-3 (35 kDa) and the 20 kDa processed (activated) form (boxed in panel a). (b). HeLa cells were either uninfected or infected with log-phase growth of GBS for 30 min. Cell lysates were either collected at this point (time 0 h), or the cell media was replaced with fresh, antibiotic containing media and 10 μM CPT was added to a subset of the cells. The cells were further incubated for 3 or 18 hours, and then the cell lysates were collected and processed as in (a).

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Chapter 7:

Summary and Future Directions

The Group B *Streptococcus* (GBS) has remained an important human pathogen despite the advances that have been made in prevention of infections caused by this bacteria. Invasive GBS disease continues to be a problem in neonates, pregnant women, and non-pregnant adults (Farley *et al.*, 1993;Schrag *et al.*, 2002;Schrag *et al.*, 2000;Tyrrell *et al.*, 2000).

For humans, the epithelial barrier is a first line of innate immunity against infection. The ability of GBS to invade the epithelial cell barrier is critical to the invasive disease process. Thus, the goal of this thesis research has been to explore GBS-host cell interactions at the molecular level. The objective of my thesis work has been to increase understanding of the host cell and bacterial factors that may be involved in actin recruitment and manipulation by GBS, as this process is known to be essential for epithelial cell invasion to occur. The assays performed during this work have focused on: (1) Characterization of host cell signal transduction processes that GBS usurp/parasitize during the epithelial cell invasion process, and (2) the role that surface-associated GBS phosphoglycerate kinase (PGK) plays in GBS pathogenesis. This work was carried out with the idea that better understanding of the infectious process of a pathogen may lead to options for therapeutics targeted at the host-pathogen interface, and ultimately increase the probability of developing novel and effective means of therapy and prevention for GBS disease in the future.

My thesis work began by establishing a working epithelial cell model for GBS invasion; in Chapter 2, I assayed the ability of several different GBS strains to invade HeLa, HEp-2, and A549 cells. Each of these cell lines is well characterized in the literature and has been utilized in previous studies examining streptococcal invasion (Greco *et al.*, 1995;Hulse *et al.*, 1993;Lalonde *et al.*, 2000;Rubens *et al.*, 1992;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997). Typically A549 (lung epithelial) cells are considered the neonatal model and HeLa (cervical epithelial) cells the maternal model of GBS colonization/invasion. A serotype V clinical strain from a soft tissue wound (Tyrrell *et al.*, 2002) invaded each of these epithelial cell types. Once this working model for invasion was established, I was subsequently able to perform manipulations to the system to establish the involvement of certain bacterial and host cell factors in the invasion process.

7.1. Phosphoglycerate Kinase in GBS Pathogenesis.

Early in the course of my thesis research, I examined the possibility that host cell components closely associated with the actin cytoskeleton, such as α-actinin, may participate in GBS invasion concurrently with actin, or facilitate coordination of cytoskeletal rearrangements leading to GBS uptake. In the host cell, α-actinin exists as a dimer composed of two antiparallel peptides (Fig. 7.1a) and links a diverse array of cellular moieties to the actin cytoskeleton, with its primary function considered to be cross-linking actin filaments into a tetragonal lattice (Fig. 7.1b) (Djinovic-Carugo *et al.*, 1999).

α-actinin is recruited by several bacterial pathogens and participates in actin rearrangement leading to pathogen internalization into non-phagocytic host cells. For example, in the process of *FimH* mediated invasion of uropathogenic

Escherichia coli, there is a transient formation of vinculin/α-actinin complexes leading to bacterial internalization (Martinez *et al.*, 2000). It is thought that α-actinin stabilizes the zipper-type actin rearrangement that occurs during the invasion of this pathogen (Martinez *et al.*, 2000). *Listeria monocytogenes* is another example of a pathogen that manipulates host cell α-actinin (Nanavati *et al.*, 1994). *L. monocytogenes* escapes from the phagolysosome into the host cell cytoplasm after invasion (Cossart *et al.*, 2003) and once in the cytoplasm, *L. monocytogenes* has the ability to induce actin polymerization, and actin filaments form into projections or "tails" behind the intracellular organisms (Cossart *et al.*, 2003;Nanavati *et al.*, 1994). *L. monocytogenes* is able to regulate the incorporation of α-actinin into these tails, and this modulation of α-actinin seems to correlate with the speed and direction of movement of the bacterium through infected cells (Nanavati *et al.*, 1994). Thus, α-actinin mediated bacterial invasion is a somewhat common theme in bacterial pathogenesis, therefore α-actinin was thought to be a logical choice to pursue for the initial investigations into cytoskeletal manipulations induced by GBS during the host-cell invasion process.

Initially, α-actinin antibodies were used to assay for localization of α-actinin to the site of GBS attachment and entry into epithelial cells. However, it was observed that rather than GBS recruiting α-actinin, the antibodies bound to the surface of GBS. At first, this finding seemed puzzling, however, it is a good example of how science continues to surprise us. This is on a similar theme to the recent "surprise" discovery of GBS pili (Buccato *et al.*, 2006;Lauer *et al.*, 2005), which defied the long-standing dogma that Streptococci do not possess pili. Thus, alternative explanations were considered.

In this case, binding of α-actinin antibodies to GBS implied the presence of an actin binding protein on the GBS surface. One-dimensional Western blot and subsequent tandem mass spectroscopy analysis identified phosphoglycerate kinase (PGK) as one potential actin-interacting protein on the GBS cell surface. Although the initial evidence leading to PGK was somewhat circumstantial in nature, the work that followed clearly demonstrated a role for PGK in actin manipulation and GBS pathogenesis. The addition of PGK to epithelial cells prior to infection with GBS inhibited GBS invasion, and also disrupted the actin stress fiber pattern of the cells. In contrast, mixing whole GBS with filamentous actin resulted in macroscopic agglutination that, upon microscopic analysis, resembled a lattice-type array. These data together suggested that the attachment of PGK to a solid support, such as the GBS surface, is important for agglutination to occur. When PGK in solution is added to epithelial cells, or PGK is expressed inside of the cells, it is not attached to a matrix or solid structure to facilitate an ordered array. Thus, rather than recruiting actin filaments in an ordered fashion, the interaction of soluble PGK with the cytoskeleton abrogates the normal actin stress fiber pattern in the cell. This suggests that in the agglutination observed in the GBS/filamentous-actin reaction mixture, or in the recruitment of filamentous actin to the site of GBS attachment and entry, the chains of GBS may "replace" α-actinin in the tetragonal lattice during their interactions with the epithelial cell cytoskeleton (Fig. 7.1c).

During the assays where PGK in solution was added to epithelial cells or expressed inside of epithelial cells, dramatic rearrangements to the actin cytoskeleton were observed. The extent of cytoskeletal alteration suggested that the effect of PGK on these cells may be more extensive than simply binding to the cytoskeleton, and possibly that PGK may have the ability to modulate host cell signal transduction pathways that participate in cytoskeletal remodeling. This has been demonstrated to occur for other glycolytic enzymes involved in pathogenesis, such as the Group A Streptococcal Surface Dehydrogenase (SDH) (Boel *et al.*, 2005;Pancholi and Fischetti, 1992;Pancholi and Fischetti, 1993). The host-cell signal transduction pathways leading to in bacterial internalization have been well characterized for several pathogens, such as *Salmonellae* and *L. monocytogenes* (Ahmadian *et al.*, 2002;Cossart *et al.*, 2003;Finlay and Cossart, 1997;Galan and Zhou, 2000;Gruenheid and Finlay, 2003;Ireton *et al.*, 1999;Lesser *et al.*, 2000). However, a review of the literature on GBS invasion revealed that host cell signal transduction events occurring in GBS invasion of epithelial cells were largely uncharacterized, with the main exception being that phosphoinositide-3 kinase (PI3K) may be involved in this process (Tyrrell *et al.*, 2002). Thus, the next phase of my thesis research evolved into characterization of host cell signal transduction events involved in epithelial cell invasion by GBS, with the idea that examination of host cell moieties and signal transduction pathways involved in invasion may identify possible target molecules for PGK, and subsequent modulation of these components by PGK could be assayed.

7.2. Host cell signal transduction pathways in GBS invasion.

To study signaling pathways involved in GBS invasion, the epithelial cell invasion process was mimicked using HeLa cell culture in conjunction with chemical inhibitors to, and dominant negative forms of, cell signaling molecules commonly involved in bacterial invasion of host cells. The work in this thesis demonstrates that the PI3K/Akt signaling pathway and the Rho-family GTPases are key players in GBS invasion (Chapters 5 and 6) and that they may be regulated upstream by tyrosine kinase activation (Appendix I). The Rho-family GTPases are central regulators of cytoskeletal organization and cellular morphology in eukaryotic cells (Hall, 1998). PI3K is a lipid kinase that catalyzes the addition of a phosphate to phosphoinositides; the resulting phospholipids molecules modulate the actin cytoskeleton with precise spatial and temporal control (Stokoe, 2005;Vanhaesebroeck and Alessi, 2000). The result of manipulation of this pathway by a pathogen is coordination of actin rearrangement, leading to internalization of the organism. In the eukaryotic cell, the PI3K and Rho GTPase pathways can converge to trigger cytoskeletal rearrangement and bacterial uptake. While it is established here that each pathway is important in GBS invasion in its own right, interplay between these two pathways leading to GBS uptake is also possible. Thus, although all aspects of GBS induced signal transduction are not yet known, a general picture of the events involved in GBS invasion of epithelial cells is emerging based on the work in this thesis (Fig. 7.2).

7.3. GBS trigger pro-survival signaling in epithelial cells at early time points post infection.

Apoptosis, the process of regulated cell death, is exceedingly complex and involves interplay between several molecules. It is regulated at many levels. In the eukaryotic cell, there is a constant balancing act between anti-apoptotic (survival) signals and pro-apoptotic (death) signals (Fig. 7.3) (Gao and Abu, 2000;Gao and Kwaik, 2000;Urnowey *et al.*, 2006;Weinrauch and Zychlinsky, 1999). Withdrawal of survival signals shifts this balance in the cell and the result is cell death.

There are several surface receptors on mammalian cells with the ability to transduce apoptotic signals and thus apoptosis can be mediated by death receptors on the cell surface. Apoptosis can also be initiated from inside the cell via the intrinsic pathway involving the mitochondria. Apoptosis is characterized by numerous biochemical events, such as protein kinase C activity, increase in intracellular calcium ion concentration, and caspase activation (caspases are synthesized as zymogens and become activated by proteolytic cleavage) (Ulett *et al.*, 2005;Weinrauch and Zychlinsky, 1999). Some pro-survival genes, such as Bcl-2 and Bcl-x_L are regulated both at the level of transcription and via post-transcriptional modifications, such as phosphorylation (Weinrauch and Zychlinsky, 1999).

Many intracellular pathogens have evolved mechanisms to modulate apoptosis of infected mammalian cells (Gao and Abu, 2000;Gao and Kwaik, 2000;Weinrauch and Zychlinsky, 1999). To do so, they may interact with components of the host cell apoptotic pathway directly, or interfere with regulation of transcription factors involved in apoptosis. For example, some bacterial toxins can insert pores into the mitochondria, resulting in leakage of cytochrome c, leading to activation of caspase-9 and subsequently apoptosis (Gao and Abu, 2000;Gao and Kwaik, 2000;Weinrauch and Zychlinsky, 1999). Bacterial modulation of apoptosis has several advantages. Inhibiting apoptosis may be used to subvert host defenses or to maintain a protected niche. Some pathogens induce apoptosis in one cell type (such as macrophages) but not other cell types (such as epithelial cells), and some pathogens also exhibit tissue-specific modulation of apoptosis, although the mechanism of this differential regulation is not clearly understood (Gao and Abu, 2000;Gao and Kwaik, 2000). Investigation into modulation of apoptosis by bacterial pathogens has been a hot topic of recent study as it is a mechanism for virulence employed by several pathogens such including *Salmonellae*, *Shigellae*, and Mycobacteria, just to name a few (Gao and Abu, 2000;Gao and Kwaik, 2000).

In 2000, Fettucciari and colleagues found that GBS induce apoptosis in murine macrophages and monocytes, but not in any other cell types assayed (such as the human T-cell leukemia cell line JURKAT or the human B-cell lymphoma cell line RAJI) (Fettucciari *et al.*, 2000). These investigators also found that treatment of the macrophages with inhibitors to caspase-1 and -3 did not affect the ability of GBS to induce macrophage apoptosis, and suggested that GBS triggers macrophage apoptosis via a different mechanism, either through a caspase independent pathway or using caspases other than caspase-1 or –3 (Fettucciari *et* *al.*, 2000). In 2005, Ulett *et al.* sought to further delineate the mechanisms of GBS induced macrophage apoptosis, and found that activation of caspase-3 was essential to GBS induced apoptosis, and that caspase-9 also participates, but that caspase-8 does not (Ulett *et al.*, 2005). The apparent discrepancy between these two investigations suggests there may be redundancy in GBS-induced apoptotic mechanisms, or that multiple apoptotic pathways may participate. During my investigations into the role of the PI3K/Akt signaling pathway in GBS invasion of epithelial cells, I found that, in addition to a requirement for Akt in GBS invasion, Akt is phosphorylated in response to GBS infection (Chapter 6). In addition to cytoskeletal regulation, Akt participates in the balancing act between host cell survival and cell death as a mediator of apoptosis (with the phosphorylated from of Akt being an anti-apoptotic signal in the host cell) (Fig. 7.3), and can modulate the phosphorylation state of downstream effectors that also contribute to the host cell apoptotic balance (Chan *et al.*, 1999;Datta *et al.*, 1999). In response to this finding, I explored the modulation of GSK-3 phosphorylation in GBS infection (Embi *et al.*, 1980;Pap and Cooper, 1998). It was observed that GBS infection of epithelial cells results in phosphorylation (inactivation/pro-survival signal) of GSK-3; this phosphorylation occurs quickly after the epithelial cells are infected with GBS and persists for at least 60 min post infection. Thus, two pro-survival signals induced by GBS at early time points post infection have been identified. Further, it was found that GBS are able to protect epithelial cells from chemically-induced caspase-3 cleavage for at least 18 h post infection, suggesting a possible mechanism for GBS persistence inside epithelial cells for relatively long periods of time (Greco *et al.*, 1995). Thus, the work in this thesis illustrates GBS modulation of epithelial cell components participating in the "survival" side of the epithelial cell apoptotic balance at early time points post-infection (Fig. 7.3).

7.4. Looking to the Future.

At the commencement of the research in this thesis, the molecular events leading to actin manipulation and subsequent bacterial uptake in the GBS-epithelial cell invasion process were largely uncharacterized. A picture for GBS invasion is beginning to emerge based on this thesis work and has laid a foundation for three separate, but highly linked, future investigations.

7.4.1. Group B Streptococcal Phosphoglycerate Kinase.

This thesis demonstrates that the addition of PGK to epithelial cells prior to GBS infection inhibits GBS invasion, and that PGK interacts with the epithelial cell cytoskeleton. What remains unknown is which part or parts of the PGK protein are responsible for these events. It has been demonstrated (Chapter 4) that modification of the C-terminus of PGK by the addition of a hydrophobic peptide sequence abolished GBS-PGK-mediated cytoskeletal disruption. This suggested that either the C terminus of the PGK protein itself (possibly the C terminal lysine residue) is involved in PGK-cytoskeleton interactions, or that disruption of the C-terminus abrogates a component of PGK protein folding essential to create the actin-interacting domain. Future studies in which fragments of the GBS PGK gene are expressed as GFP fusion proteins inside the epithelial cell and expression of polyhistidine tagged fragments of PGK should serve as valuable tools to address this question. Once it is determined if a particular portion of the PGK protein is responsible for disruption of the actin cytoskeleton or inhibition of invasion, this region would be an excellent target for site-directed mutagenesis studies on PGK to determine the specific residues involved.

Another question that remains for PGK (and for the other surface-associated glycolytic enzymes involved in Streptococcal pathogenesis) is how does PGK come to be localized on the GBS cell surface? Some organisms possess more than one PGK gene encoding distinct isoforms, such as *Trypanosoma brucei*, in which the *PGKB* gene encodes a cystolic form of PGK and the *PGKC* gene encodes a glycosomal PGK (Colasante *et al.*, 2007). In contrast, sequencing of several GBS genomes reveals only one chromosomal copy of the GBS PGK gene (Glaser *et al.*, 2002;Tettelin *et al.*, 2002). One possible explanation is that GBS PGK is post-translationally modified/processed in multiple ways, such that although there is only one PGK gene, there are ultimately two different PGK species in the cell—one that functions in glycolysis, and the other a surface-associated form that contributes to GBS virulence. In general, Streptococcal glycolytic enzymes have been found to be essential genes, and thus a traditional approach of creating an isogenic knock-out mutant to explore the role of the surface-associated forms of these proteins is not possible (Pancholi and Chhatwal, 2003). Future creation of a GBS mutant in which PGK is modified such that it is sequestered inside the bacterial cell and does not become surface exposed (and thus is not able to participate in interactions with epithelial cells) may be helpful in further characterization of the role of PGK in GBS pathogenesis. This approach has been useful in exploring the role of GAS SDH (Boel *et al.*, 2005).

Another question for future exploration is the potential ability of GBS PGK to interact with host plasmin(ogen). Plasminogen is a highly abundant, circulating enzyme in eukaryotes that becomes converted to plasmin by specific activators (Lahteenmaki *et al.*, 2005). The mammalian plasminogen-plasmin system is a key player in fibrinolysis and extracellular matrix degradation (Lahteenmaki *et al.*, 2005). Expression of a plasminogen receptor on the bacterial surface is a common theme in pathogenesis; several different organisms bind to plasminogen and/or plasmin, and a number of pathogens have developed mechanisms to interfere with the host plasminogen system, generally as a means to facilitate crossing host barriers (mediated by tissue degradation), or to participate in nutrient acquisition (Bergmann *et al.*, 2004;Crowe *et al.*, 2003;Fox and Smulian, 2001;Lahteenmaki *et al.*, 2005;Pancholi and Fischetti, 1998;Winram and Lottenberg, 1996). One mechanism of exploitation of this host cell system by a bacterial pathogen is the use of plasminogen as a receptor for adhesion to host tissues (Lahteenmaki *et al.*, 2005). For example, PGK on the surface of *Candida albicans* has been demonstrated to be a plasmin(ogen) binding protein (Crowe *et al.*, 2003). Both GAPDH and α-enolase on the surface of Group A *Streptococcus* have the ability to bind plasmin (Pancholi and Fischetti, 1998;Winram and Lottenberg, 1996) and α-enolase on the surface of *Streptococcus pneumoniae* also binds plasmin (Bergmann *et al.*, 2001). The presence of a plasmin(ogen) binding protein is a common theme in bacterial pathogenesis, but a plasmin(ogen) binding protein has not been described for GBS. This, in combination with the fact that PGK on the surface of other organisms has been demonstrated to function as a plasmin(ogen) binding protein, suggests that investigation into potential plasmin(ogen) binding of GBS PGK warrants further study.

A final point regarding GBS PGK is that preliminary evidence has suggested that GBS PGK is immunogenic (Hughes *et al.*, 2002). This, in combination with PGK being localized on the GBS surface and conserved amongst GBS isolates makes it an enticing vaccine target. In the future, the inclusion of PGK in a multi-part GBS vaccine should be considered (for example, in combination with components of GBS pili (Buccato *et al.*, 2006)) to provide coverage against a broad spectrum of GBS strains.

7.4.2. Modulation of host cell signal transduction pathways.

The investigations in this thesis clearly demonstrate that the PI3K/Akt signaling pathway and the Rho family GTPases are involved in the GBS invasion process. What remains somewhat unclear is tyrosine phosphorylation events in GBS invasion. Treatment of epithelial cells with genistein or sodium orthovanadate prior to GBS infection suggested a requirement for modulation of tyrosine phosphorylation in GBS invasion, but the specific host cell components that are tyrosine phosphorylated in this process were not identified. One possible reason for this was the high background level of tyrosine phosphorylation observed in the control cells. Use of poly-L-lysine coated dishes in future assays may be worthwhile as in some instances this has been shown to reduce background tyrosine phosphorylation levels (Ozeri *et al.*, 2001), and thus may reveal subtle phosphorylation events more clearly.

Immunoblot analysis for tyrosine phosphorylation in response to GBS infection did reveal modulation of a protein species with an approximate molecular weight of 130 kDa. In response to this, a host cell protein candidate worthy of further study is focal adhesion kinase (FAK). FAK is a tyrosine kinase with a molecular weight of 125 kDa, and is a PI3K binding partner involved in coordinating the assembly of complexes mediating cytoskeletal changes. Further, FAK has been previously demonstrated to be tyrosine phosphorylated in response to infection with serotype III GBS in a human brain microvascular endothelial cell line, and transfection of brain microvascular endothelial cells with a dominant negative form of FAK reduced GBS invasion by over 50% (Shin *et al.*, 2006). Shin and colleagues suggest that endothelial cell FAK participates in GBS translocation through the blood brain barrier and contributes to development of meningitis (Shin *et al.*, 2006). Thus, it is possible FAK is the protein species exhibiting modulation of phosphorylation in the epithelial cell immunoblot.

One other major question regarding GBS induced modulation of host cell signal transduction is which component(s) on the GBS cell surface trigger these events. PGK is one GBS protein worthy of further study in this context and could be assayed using the model systems developed in this thesis.

7.4.3. Modulation of epithelial cell apoptosis by GBS.

In Chapter 6, it was demonstrated that Akt and GSK-3 phosphorylation during GBS infection of epithelial cells tips the host cell balance towards anti-apoptotic, pro-survival events. However, modulation of apoptosis is a complex process, and additional downstream effectors of Akt may also be involved.

NFKB is often implicated in pathogen-mediated inhibition of apoptosis in infected cells; one of the key events for this to occur is the phosphorylation of IKB by IKB kinase which releases suppression of the transcription factor NFKB (Fig. 7.3). Modulation of IKB phosphorylation in response to GBS infection could be assayed, as well as modulation of other host-cell signals commonly associated with pathogen mediated manipulation of host cell apoptosis, such as BAD. These assays may contribute to finalizing the picture of GBS mediated inhibition of epithelial cell apoptotic events at early time points post-infection. Further, identification of the specific bacterial components involved in modulation of apoptosis would pose an interesting target for future study and creation of novel therapeutic agents to combat GBS disease.

During the course of this thesis work, it was found that GBS do not induce Caspase-3 cleavage in HeLa cells up to 36 h post infection. One explanation is that GBS does not induce apoptosis in epithelial cells as it does in macrophages (Fettucciari *et al.*, 2000;Ulett *et al.*, 2005) however the evidence to date does not rule out GBS-induced apoptosis at late time points post infection. A second possibility is that apoptosis does occur, but via a caspase-independent mechanism.

Thus, future investigations should include examination of other apoptotic signaling events at late stages post infection. It is also possible that the lack of Caspase-3 cleavage in this assay system is a function of working with a transformed cell line. Future use of primary epithelial cell culture could address this possibility.

7.5. Conclusions.

While much remains to be discovered regarding GBS pathogenesis, the data contained within this thesis addresses several facets of the GBS invasion process. The host-cell signal transduction cascade leading to epithelial cell invasion by GBS is beginning to take shape, namely the role of the PI3K/Akt signaling pathway and the Rho-family GTPases in invasion. A novel function for a protein on the GBS surface, phosphoglycerate kinase, has also been identified. PGK has been found to interact with the actin cytoskeleton, and the addition of PGK to epithelial cells prior to GBS infection inhibits GBS invasion of these cells. The data is this thesis advances understanding of the mechanisms of GBS invasion at the molecular level, including events leading up to actin rearrangement and subsequent internalization by this organism.

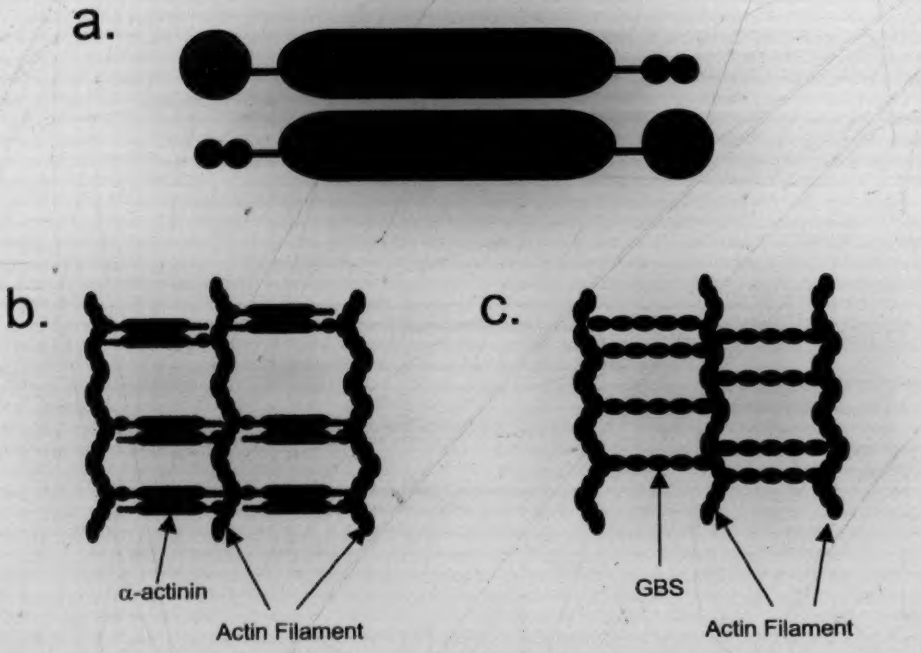

Fig. 7.1. (a). Schematic diagram of α-actinin. This protein exists as a dimer composed of two identical, anti-parallel peptides. The actin binding domain (ABD) of each subunit is near the N-terminus. (b). α-actinin acts as an actin cross-linking protein, and bundles together actin filaments into parallel or anti-parallel structures to form a tetragonal lattice (Adapted from: Djinovic-Carugo *et al.*, 1999). α-actinin antibodies bind to the surface of GBS. When GBS are mixed with filamentous actin, visible agglutination occurs. (c). The evidence in this thesis suggests that GBS possess an actin binding domain and possibly replaces α-actinin in the actin/α-actinin interaction illustrated in (b).

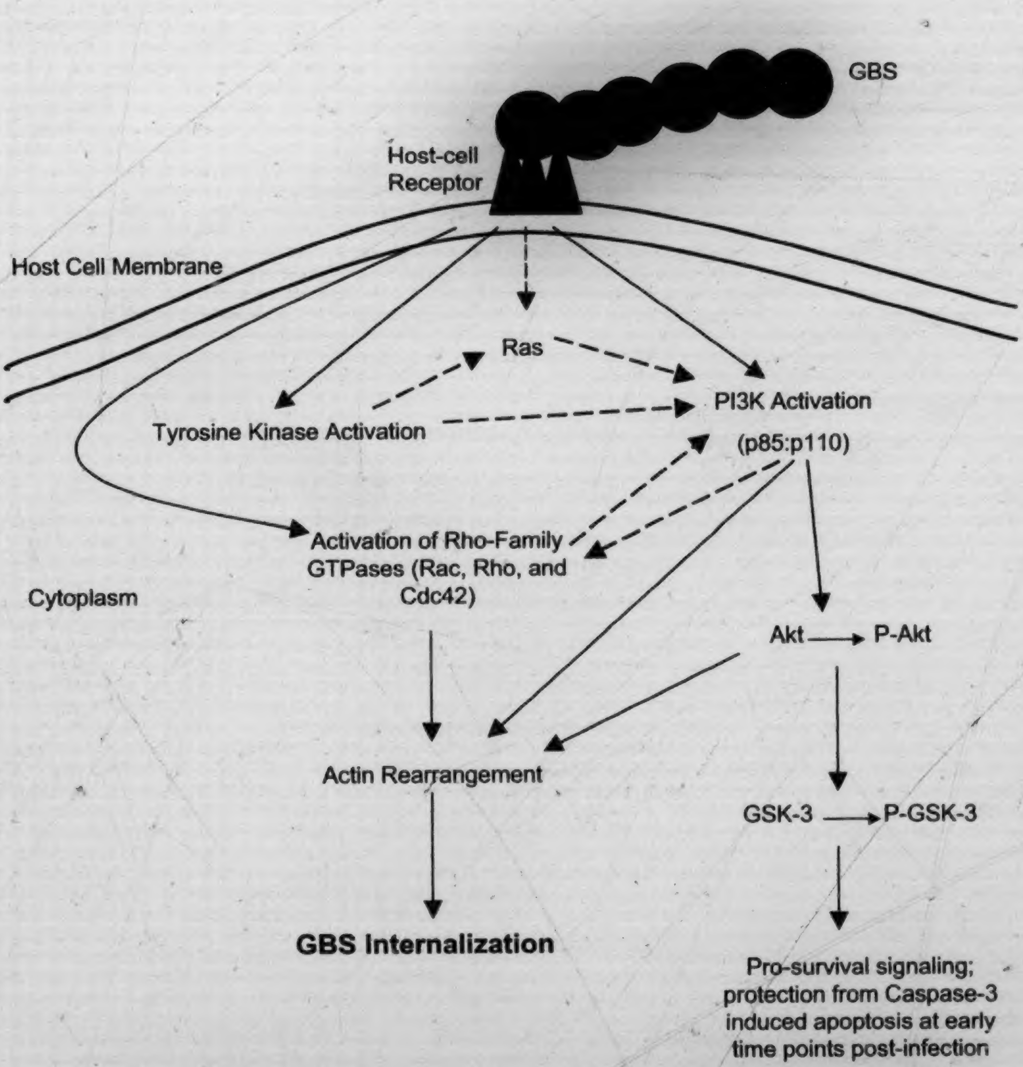
GBS
Host-cell Receptor
Host Cell Membrane
Ras
PI3K Activation
(p85:p110)
Tyrosine Kinase Activation
Activation of Rho-Family GTPases (Rac, Rho, and Cdc42)
Cytoplasm
Akt
P-Akt
Actin Rearrangement
GSK-3
P-GSK-3
GBS Internalization
Pro-survival signaling; protection from Caspase-3 induced apoptosis at early time points post-infection

Fig. 7.2. Schematic diagram illustrating the proposed host-cell signaling cascade facilitating GBS invasion into epithelial cells. GBS invasion is a complex process involving multiple host-cell signaling pathways and events. Solid arrows represent signaling events characterized in this thesis, dashed arrows represent possible signaling events and/or interactions.

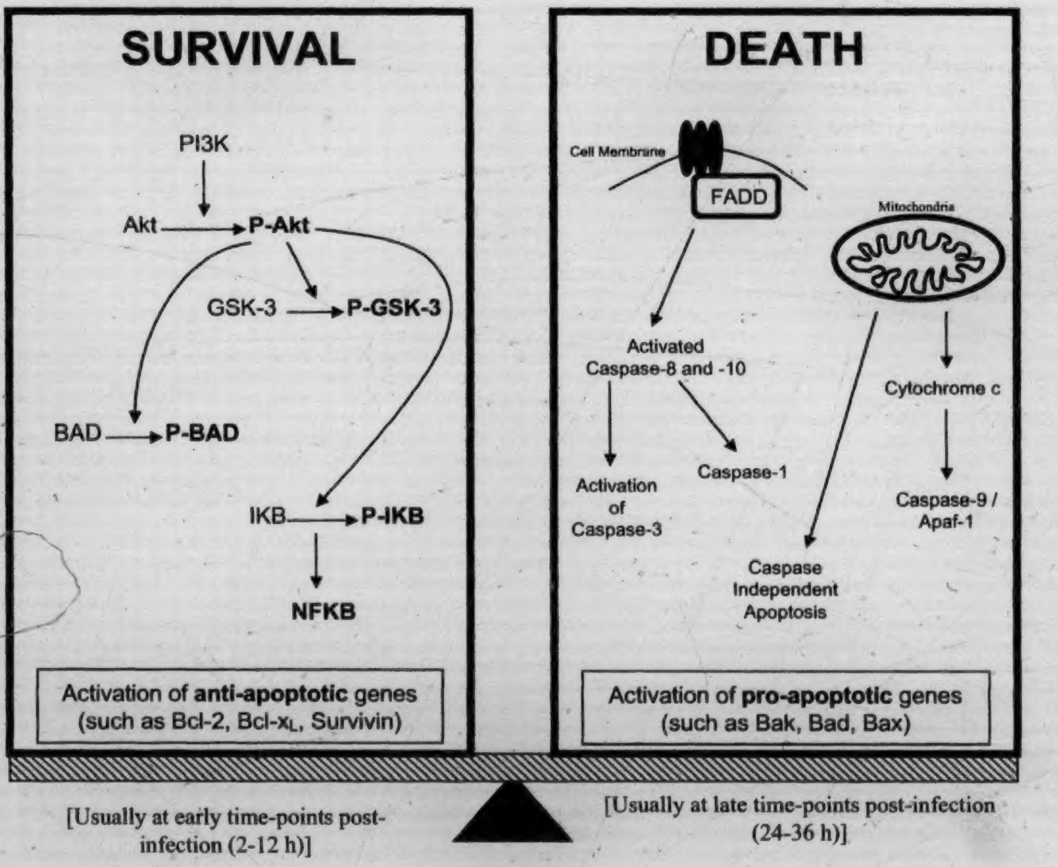
SURVIVAL
PI3K
Akt
P-Akt
GSK-3
P-GSK-3
BAD
P-BAD
IKB
P-IKB
NFKB
Activation of **anti-apoptotic** genes (such as Bcl-2, Bcl-x$_L$, Survivin)
DEATH
Cell Membrane
FADD
Mitochondria
Activated Caspase-8 and -10
Cytochrome c
Activation of Caspase-3
Caspase-1
Caspase-9 / Apaf-1
Caspase Independent Apoptosis
Activation of **pro-apoptotic** genes (such as Bak, Bad, Bax)
[Usually at early time-points post-infection (2-12 h)]
[Usually at late time-points post-infection (24-36 h)]

Fig. 7.3. Simplified schematic diagram illustrating the balancing act between pro-survival and pro-apoptotic signals in the eukaryotic cell. Apoptosis is a highly regulated process transduced through multiple pathways, some examples of which are depicted here. Invasive and intracellular bacterial pathogens have evolved a plethora of strategies to modulate host-cell apoptosis to their advantage, and promote the infectious process. Adapted from: Gao and Abu, 2000, Gao and Kwaik, 2000, and Urnowey *et al.*, 2006).

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Appendix I:

Host-Cell Tyrosine Phosphorylation During Group B Streptococcal Invasion of Epithelial Cells

I.1. Introduction

The Group B *Streptococcus* (GBS) is an opportunistic human pathogen. Disease manifestations of GBS infection include meningitis, pneumonia, sepsis and infections of bone and soft tissue. GBS is the most frequent causative agent of meningitis in infants less than one month of age in the United States (Schuchat *et al.*, 1997), with serotypes Ia, Ib and III being most strongly associated with neonatal infection. Invasive GBS disease can also occur in adults, especially in the elderly population or in those with an underlying chronic disease (Farley *et al.*, 1993;Schuchat *et al.*, 1990;Schuchat, 1998;Tyrrell *et al.*, 2000).

GBS have the ability to induce their own uptake into a variety of non-phagocytic host cell types, including epithelial and endothelial cells (Gibson *et al.*, 1993;Lalonde *et al.*, 2000;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997). While many of the specific molecular interactions between GBS and the host cell that facilitate this process remain uncharacterized, it has been established that host cell actin microfilaments are recruited to the site of GBS attachment and subsequent invasion (Gibson *et al.*, 1993;Tyrrell *et al.*, 2002;Valentin-Weigand *et al.*, 1997). Numerous pathogens have developed sophisticated mechanisms for parasitizing host cell signal transduction networks, followed by recruitment of other host components to the site of the organism's entry (Criss and Casanova, 2003;Finlay and Cossart, 1997;Ireton *et al.*, 1999;Kwok *et al.*, 2002). The recruitment and manipulation of actin is a common end result of this process (Ahmadian *et al.*, 2002;Gruenheid and Finlay, 2003).

Host cell signaling pathways activated or recruited by invasive bacteria often involve modulation of host cell protein phosphorylation. Phosphoinositide-3 kinase (PI3K) and Rho family GTPases have been demonstrated to be involved in the GBS epithelial cell invasion process (Chapters 5 and 6); these host cell moieties function as molecular switches and can be activated by plasma membrane receptors for extracellular signals, such as receptor tyrosine kinases. The objective in this chapter was to explore the requirement of host cell tyrosine phosphorylation in GBS invasion of epithelial cells.

I.2. Materials and Methods

I.2.1. Bacterial strains, cell lines, and growth conditions.

The GBS strain NCS13 (a serotype V strain) has been previously described (Tyrrell *et al.*, 2002). The *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) control strain NCTC 10241 was obtained from the National Culture Collection, PHLS Colidale, London, England. These organisms were cultured at 35 °C on sheep blood agar plates (BAPs) (Dalynn Biologicals) or in Todd Hewitt Broth (THB) (Difco Laboratories).

The human epithelial cell line HeLa 229 (ATCC CCL-2.1) was grown in OPTI-MEM I reduced serum medium supplemented with 4% fetal bovine serum (FBS) (Gibco BRL). The human epithelial cell line HEp-2 (ATCC CCL-23) was grown in MEM supplemented with 10% FBS and 1% glutamine (Gibco BRL). Both cell lines were obtained from the American Type Culture Collection (ATCC).

I.2.2. Invasion (antibiotic protection) assay.

Invasion of bacteria into epithelial cell cultures was quantified using a standard antibiotic protection invasion assay with minor modifications (Rubens *et al.*, 1992;Tyrrell *et al.*, 2002). The assay system has been used extensively to investigate the streptococcal invasion process (Ozeri *et al.*, 2001;Purushothaman *et al.*, 2003;Shin and Kim, 2006;Valentin-Weigand *et al.*, 1997). Briefly, cell culture monolayers were grown to confluence in 24 well plates, and then treated with chemical inhibitors or DMSO (control cells) for 1 h prior to bacterial infection. Genistein was purchased from Calbiochem and Sigma-Aldrich, genistin from Calbiochem, and sodium orthovanadate from Sigma-Aldrich. GBS or *S. typhimurium* were grown in THB with agitation overnight. A 0.5 McFarland standard of the organism was made in THB and then diluted 1 in 100 in THB. For GBS, 100 μL of this suspension (approximately 1.5 X 10^5 bacteria), and for *S. typhimrium*, 25 μL of this suspension (approximately 4 X 10^4 bacteria) was applied to the cell monolayer. The difference in inoculum is due to the fact that *S. typhimurium* invades with higher efficiency than does GBS. Following inoculation, the plates were centrifuged at 100 *g* for 5 min at room temperature. After a 2 h incubation at 35 °C to allow internalization, the monolayer was washed three times with phosphate buffered saline (PBS) to remove unbound bacteria.

Any bacteria that had bound but not internalized were killed by incubation for 2 h with fresh media containing 5 µg of penicillin per mL and 100 µg of gentamicin per mL. After this incubation, an aliquot of the media was applied to a BAP to ensure that all extracellular bacteria had been killed. The monolayers were washed with PBS, trypsinized, and lysed with 0.1% Triton X-100. The lysates were spread onto BAPs and incubated overnight at 35 °C. The number of colonies on each plate were counted to determine the number of CFU that had invaded the monolayer.

I.2.3. Statistical analysis.

For the invasion assays, relative percent invasion was calculated as follows: [(Number of CFU invaded into treated cells/Number of CFU invaded into control cells) X 100%]. Each assay condition was performed in triplicate. GraphPad InStat3 software was used for statistical analysis of the data; analysis of variance using Dunnet's Multiple Comparisons Test was used to compare invasion into inhibitor treated cells to that observed in the control cells. A P value < 0.05 was deemed to be statistically significant and is denoted as "*" in the graphical representations of data. The graphical representations are illustrative of the mean of one representative experiment; error bars represent standard error of the mean (SEM).

I.2.4. Attachment assay.

HeLa cells were grown to confluence on glass coverslips in shell vials, and either left untreated or treated with 50 μM genistein for 1 h prior to bacterial infection. GBS was grown in THB overnight; the following day, this was adjusted to a 0.5 McFarland standard and diluted 1 in 100 in THB. 100 μL of this suspension (approximately 1.5 X 10^5 bacteria) was added to the HeLa monolayer and the shell vials were centrifuged for 5 min at 100 *g*. After 2 h incubation to allow bacterial attachment, the monolayer was washed three times with PBS to remove unbound bacteria. The cells were fixed for 30 min with 3% formaldehyde. To visualize bound GBS, the preparations were stained with rabbit polyclonal anti-GBS antisera diluted 1:1000 (Difco Laboratories) followed by anti-rabbit FITC conjugate (Sigma-Aldrich) diluted 1:50. To visualize the epithelial cells, a 1:40 dilution of Alexa Fluor 568 labeled phalloidin (Molecular Probes) was used. The coverslips were mounted onto glass slides, and visualized using a fluorescence microscope (Olympus Microscopes, Carsen Group Inc.).

I.2.5. Immunoblotting.

To assess tyrosine phosphorylation in response to GBS infection, HeLa cells were grown in 6 well culture plates to 85 to 95% confluence and then infected with 200 μL of a 3.0 McFarland standard of GBS (corresponding to approximately 1.8 X 10^8 bacteria). The culture plates were centrifuged at 100 *g* for 5 min at room temperature and then incubated at 35 °C for 1, 5, 10, 20 or 30 min. The cells were then washed in PBS, and harvested in 1 X Cell Lysis Buffer (Cell Signaling Technology) supplemented with 0.2% Protease Inhibitor Cocktail for Mammalian Cell Extracts (Sigma-Aldrich) and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma-Aldrich). The cell lysates were centrifuged at 13 000 *g* for 10 min. The protein concentration of the supernatant fraction was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories), which is based on the method of Bradford (Bradford, 1976). One hundred μg of cell lysate per assay condition was resolved via SDS-PAGE using a 10% separating gel, and electroblotted to a 0.45 μM nitrocellulose membrane (Bio-Rad) following standard protocols (Bollag and Edelstein, 1991;Towbin *et al.*, 1992). Anti-phosphotyrosine clone 4G10 antibody (Upstate Cell Signaling Solutions) was used according to manufacturer's instructions to probe for tyrosine phosphorylated host-cell proteins, decorated with alkaline-phosphatase conjugated secondary antibody, and detected using the Amersham Biosciences Enhanced Chemiluminescence (ECL) system and Biomax light film for Chemiluminescence (Kodak). An EGF-stimulated A431 cell lysate (Upstate Cell Signaling Solutions) was used as a positive tyrosine phosphorylation control.

For phosphotyrosine immunoprecipitation analysis, HeLa cells were infected and the lysates collected as described for the immunoblot analysis, then 1 μg of anti-phosphotyrosine clone 4G10 antibody was added to 100 μL of cell lysate. This mixture was incubated at 4 °C for 3 h with agitation, followed by the addition of 50 μL of Protein G-Sepharose suspension (Sigma-Aldrich) and incubated overnight at 4 °C with agitation. The beads were washed, boiled in 50 μL Laemmeli sample buffer and separated by 10% SDS-PAGE with immunoblotting performed as described for tyrosine phosphorylation.

I.3. Results

I.3.1. Inhibition of host cell tyrosine kinase activity reduces GBS invasion into epithelial cells.

To determine if tyrosine phosphorylation is required for GBS entry, HeLa cells were treated with genistein, a broad-spectrum inhibitor of tyrosine kinases (Akiyama *et al.*, 1987), prior to bacterial infection. At a concentration of 50 μM, genistein reduced GBS entry by over 90%, and at 100 μM genistein, GBS invasion was nearly abolished (Fig. I.1a). *S. typhimurium*, in comparison, was still able to invade HeLa cells at a genistein concentration of 100 μM (approximately 70% internalization) (Fig. I.1a), indicating that invasion of HeLa cells by *S. typhimurium* was not as sensitive to treatment with genistein as was GBS.

Previous investigators have shown that genistein inhibited the entry of Group A *Streptococcus* (GAS) into HeLa cells, but failed to inhibit invasion into HEp-2 cells (Ozeri *et al.*, 2001;Purushothaman *et al.*, 2003). In addition to use of different cell lines, these reports used different genistein preparations (one from Sigma-Aldrich, the other from Calbiochem). To rule out the likelihood that variation between genistein preparations could influence GBS internalization differently, both cell types were assayed using both preparations of genistein. The effect of genistin, an inactive analogue of genistein, was also assayed for both cell types.

GBS internalization was inhibited by the genistein treatment in both HeLa and HEp-2 cells (Fig. I.1b). Both preparations of genistein inhibited GBS internalization, however, HeLa cell invasion was more sensitive to the effects of genistein than HEp-2 invasion (Fig. I.1b). The Calbiochem preparation appeared to be slightly more potent at inhibiting invasion at the concentrations assayed than the Sigma-Aldrich preparation; at 50 μM, Sigma-Aldrich genistein reduced GBS invasion by approximately 85% in HeLa cells, and 65% in HEp-2 cells (Fig. I.1b). At the same concentration, Calbiochem genistein reduced GBS invasion of HeLa cells by over 90% and HEp-2 invasion by over 75% (Fig. I.1b).

Epithelial cell treatment with genistin prior to GBS internalization resulted in a slight decrease in GBS invasion of HeLa cells (Fig. I.1b). Why this occurred is not clear, but the reduction in invasion is not statistically significant (*P* value > 0.05 as compared to untreated control cells). HEp-2 invasion by GBS was unaffected by the genistin treatment (Fig. I.1b).

I.3.2. Treatment of HeLa cells with genistein does not reduce GBS attachment.

In order to confirm that the genistein treatment was not affecting GBS adhesion to HeLa cells, a microscopic attachment assay was performed comparing

GBS attachment to untreated cells (Fig. I.2a) and cells treated with 50 μM genistein for 1 h prior to GBS infection (Fig. I.2b). A minimum of 10 microscopic fields were examined for each treatment condition, and a representative example for each is shown in Fig. I.2. No difference in the amount of GBS attachment could be detected visually between the two assay conditions.

I.3.3. Inhibition of host cell tyrosine phosphatases modulates GBS invasion of HeLa cells.

Sodium orthovanadate is a protein tyrosine phosphatase inhibitor that is presumed to function by acting as a phosphate analogue (Gordon, 1991;Stankiewicz and Gresser, 1988). The observation that tyrosine kinase inhibition resulted in a marked reduction in GBS invasion suggested that phosphorylation of host cell proteins is an important factor in GBS invasion of host cells. Thus, I postulated that the opposing process, inhibiting protein tyrosine phosphatases, might also modulate GBS invasion, and possibly enhance the invasion process. I exposed HeLa cells to 10, 30, 100, and 300 μM of sodium orthovanadate for 1 h prior to GBS and *S. typhimurium* infection and compared the amount of internalization to that into control cells. I observed that at the low sodium orthovanadate concentration of 10 μM, GBS invasion was enhanced by nearly 40%, although this finding was not statistically significant (Fig. I.3). At the intermediate sodium orthovanadate concentrations of 30 and 100 μM, no change in GBS invasion was observed, and at the high concentration of 300 μM, GBS invasion was reduced by approximately 50% (Fig. I.3). GBS invasion into HeLa cells treated with 50 μM genistein or genistin was assayed in parallel as a control; as expected, genistein treatment significantly reduced GBS invasion and genistin did not affect invasion (Fig. I.3). The invasion of *S. typhimurium* was not significantly affected by sodium orthovanadate at any concentration assayed (Fig. I.3).

I.3.4. Host cell tyrosine phosphorylation in response to GBS infection.

The inhibitory effect of genistein on GBS invasion of epithelial cells implicated host cell protein tyrosine phosphorylation as a mediator of the GBS invasion process. To examine tyrosine phosphorylation of a particular host cell protein or proteins in response to GBS infection, Western blot analysis was performed on lysates of GBS infected HeLa cells at 1, 5, 10, 20, and 30 min post infection. Examination of the immunoblot revealed a decrease in phosphorylation in a protein species with an approximate molecular weight of 130 kDa at 1 min post infection (Fig. I.4a). At 5 min post infection, the phosphorylation level appears to return to the basal level observed in untreated control cells. This illustrates modulation of host cell protein tyrosine phosphorylation in response to GBS infection, data consistent with the result that host cell treatment with genistein inhibited GBS internalization.

In light of the high background level of tyrosine phosphorylation observed in untreated control HeLa cells, it was postulated that this assay approach may not adequately detect subtle alterations in tyrosine phosphorylation levels, and thus immunoprecpitation was performed on GBS infected HeLa cell lysates using anti-phosphotyrosine antibody, in an attempt to enhance the likelihood of detecting tyrosine phosphorylated host cell proteins. However, the immunoblot of the immunoprecipitates did not yield any further information (Fig. I.4b).

An immunofluorescence assay of GBS infected HeLa cells was also performed using the anti-phosphotyrosine antibody. No recruitment of tyrosine phosphorylated proteins around the site of bacterial attachment and internalization compared to the high level of background staining could be resolved using this assay method (not shown).

I.4. Discussion

Tyrosine phosphorylation resulting from tyrosine kinase activation is a feature that accompanies the binding to and/or invasion of several bacterial pathogens into host cells (Ozeri *et al.*, 2001;Tang *et al.*, 1994;Williams-Bouyer and Hill, 1999). In this investigation, HeLa cells were used as an infection model to explore the signal transduction processes involved between GBS and epithelial cells during invasion, with a focus on modulation of host cell tyrosine phosphorylation in response to GBS infection.

Modification of the phosphorylation state of a molecule generally results in alteration of its biochemical properties and biological activities. Reversible tyrosine phosphorylation in the host cell regulates an array of cellular functions, such as proliferation, migration, and changes to the actin cytoskeleton. Interactions between phosphorylated proteins and binding partners is often a crucial step in the execution of the biological function mediated by the alteration in phosphorylation state. Mammalian cells encode approximately 90 tyrosine kinases, nearly 60 of which are receptor tyrosine kinases and the remaining 30 are cytosolic tyrosine kinases (Robinson *et al.*, 2000).

Genistein was utilized to screen for potential involvement of tyrosine kinases in GBS invasion. GBS invasion was shown to be very sensitive to genistein treatment, suggesting tyrosine kinase involvement. An assay was also performed where HeLa cells were treated with sodium orthovanadate, a tyrosine phosphatase inhibitor, prior to GBS infection. At a low concentration, this treatment resulted in a slight increase in GBS invasion, and at a high concentration, invasion decreased by approximately 50%. This suggests that the interplay between phosphorylation and dephosphorylation events is key during GBS invasion of epithelial cells; that is, the inhibition of host-cell phosphorylation modulation is deleterious to the GBS invasion process. These findings implicate host cell tyrosine phosphorylation in the host cell signaling network involved in GBS invasion into epithelial cells.

Tyrosine phosphorylation of receptor tyrosine kinases at the host cell surface can activate PI3K by binding to the p85 PI3K subunit (Carpenter and Cantley, 1996;Kwiatkowska and Sobota, 1999). It has been shown that PI3K is involved in GBS invasion of epithelial cells, and that transfection of HeLa cells with a dominant negative form of the p85 PI3K subunit inhibits GBS invasion into this cell type (Tyrrell *et al.*, 2002; Chapter 6). The work in this investigation suggests that activation of PI3K could possibly occur downstream from host cell tyrosine kinase phosphorylation.

Shin and colleagues observed that treatment of human brain microvascular endothelial cells (HBMEC) with 100 μM genistein prior to GBS infection inhibits GBS invasion into this cell line, and that GBS infection of HBMEC induced tyrosine phosphorylation of host cell proteins (35, 65, 80, and 125 kDa in size) (Shin *et al.*, 2006). The time frame in which these phosphorylation events occur ranges from 15 to 90 min post infection (Shin *et al.*, 2006). The fact that tyrosine phosphorylation events are a feature of GBS invasion of both epithelial cells and brain endothelial cells suggests that tyrosine phosphorylation is a general feature of GBS invasion into different host cell types.

The Western blot analysis for tyrosine phosphorylation in GBS infected cells revealed modulation of phosphorylation of only one protein species in the host cell. It is very likely that other tyrosine phosphorylation and/or dephosphorylation events also occur in response to GBS infection that are not being detected in this analysis. The lack of ability to detect such events is likely due to the high background phosphorylation in untreated cells, and that events triggered in response to GBS infection are like "a drop in a bucket" in the context of this analysis. Further, is it both possible and likely that GBS induced phosphorylation events occur quickly, and are transient in nature, and thus may be difficult to capture, particularly in a static assay system.

The protein species that was demonstrated to exhibit modulation of protein tyrosine phosphorylation in response to GBS infection was that with an approximate molecular weight of 130 kDa. Focal adhesion kinase (FAK) is a tyrosine kinase with a molecular weight of 125 kDa; it is a PI3K binding partner and is involved in coordination of the assembly of signaling complexes mediating cytoskeletal changes. It has been demonstrated that FAK activity is a feature of GBS invasion into HBMEC (Shin *et al.*, 2006). It is possible that FAK is the protein species exhibiting phosphorylation modulation in the Western Blot. This could be assayed using Western blot of infected lysates with anti-FAK antibody and/or immunoprecipitation of infected lysates with anti-phosphotyrosine antibody, followed by Western blot with anti-FAK.

The ability of GBS to invade nonphagocytic cells within the host presents a wide variety of advantages to the bacterium, such as the ability to overcome unfavorable host conditions, gain access to privileged sites, or evade the host's immune system. In order to invade host cells, bacterial pathogens typically parasitize or trigger pre-existing host cell signal transduction cascades, and various pathogens have evolved different mechanisms for doing so. Most signal transduction cascades involve modification of proteins by phosphorylation. The goal in this chapter was to examine modulation of host cell tyrosine phosphorylation during GBS invasion; it was found that manipulation of host cell tyrosine phosphorylation is an essential event in GBS invasion of epithelial cells.

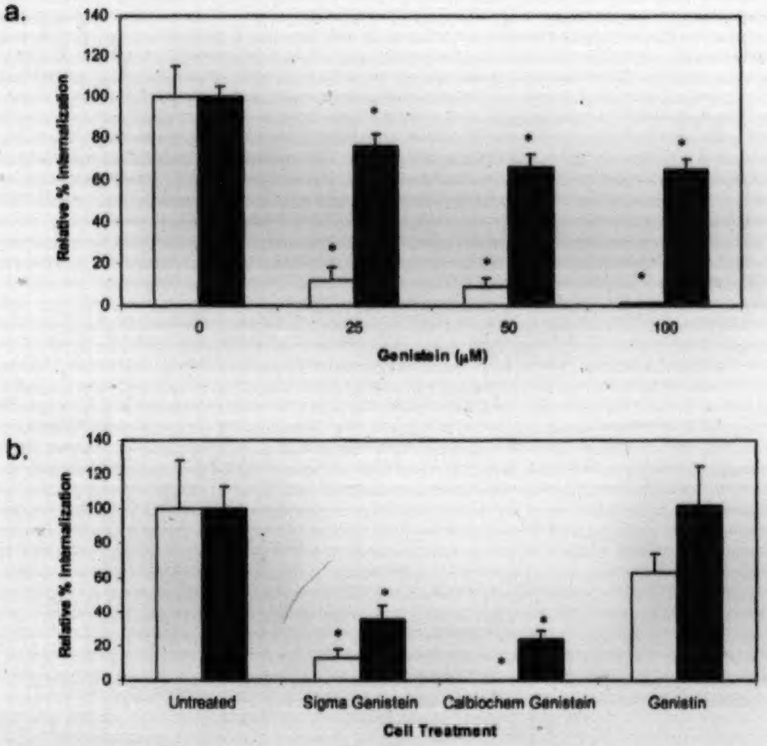

Fig. I.1. GBS invasion into epithelial cells is dependant upon tyrosine kinase activation. (a). Genistein, a broad spectrum tyrosine kinase inhibitor, was used to treat HeLa cells for 1 h prior to bacterial infection. The genistein treatment inhibited GBS invasion in a dose dependant manner. Solid bars (■), *S. typhimurium*; Open bars (□), GBS. (b). Invasion of HeLa cells (open bars, □) by NCS13 is more sensitive to treatment with 50 μM of genistein prior to bacterial infection than invasion into HEp-2 cells (solid bars, ■). Genistin, an inactive analogue of genistein, has a slight inhibitory effect on GBS invasion of HeLa cells but does not affect HEp-2 cell invasion by GBS. Relative percent internalization was calculated as follows: [(Number of CFU invaded into treated cells/Number of CFU invaded into control cells) X 100%]. Graphical representations are illustrative of the mean one representative experiment in which each assay condition was performed in triplicate. Error bars represent standard error of the mean. "*" indicates a P value < 0.05 compared to untreated control cells as evaluated using Dunnet's Multiple Comparisons Test.

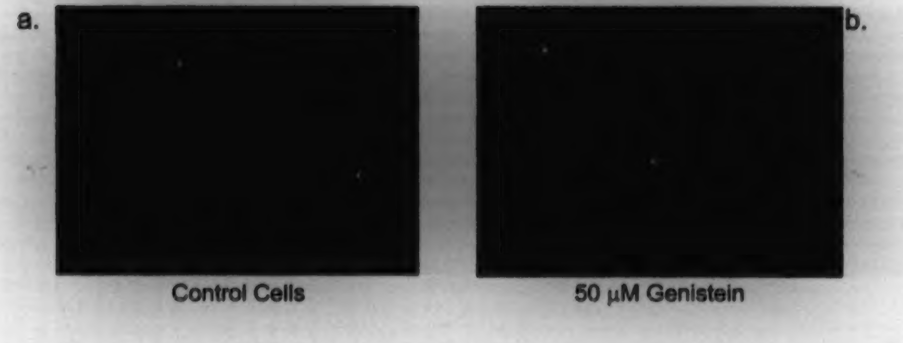

Fig. I.2. Treatment of HeLa cells with genistein does not prevent GBS attachment. HeLa cells were left untreated (a.) or treated with 50 μM genistein (b.) for 1 h prior to bacterial infection, infected with NCS13 and then fixed. Bacteria were stained using anti-GBS antibody decorated with anti-rabbit FITC conjugate. HeLa cells were stained with Alexa-fluor 568 labeled phalloidin. Arrows indicate GBS (green) attached to HeLa cells (red).

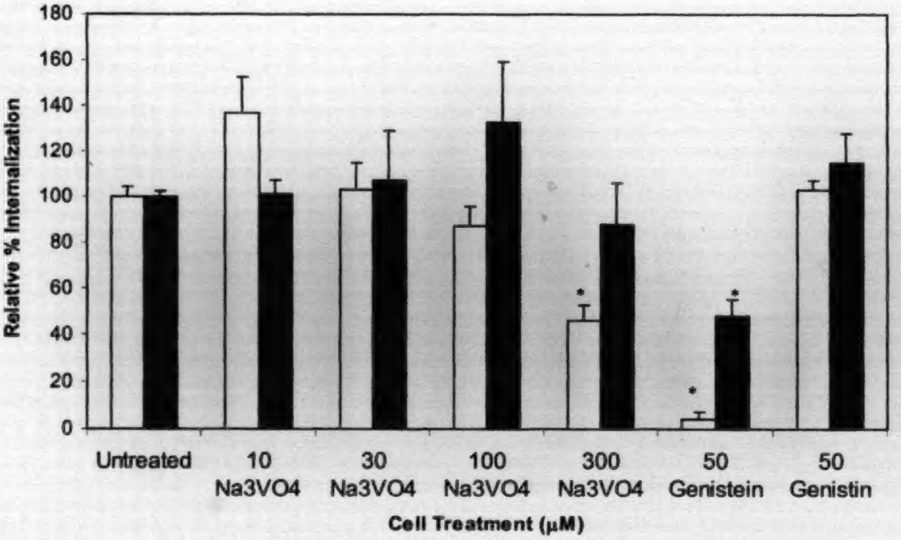

Fig. I.3. Treatment of HeLa cells with sodium orthovanadate (Na_3VO_4) prior to GBS infection enhances invasion at a low concentration, and inhibits invasion at a high concentration. Invasion of *S. typhimurium* is not altered in response to the Na_3VO_4 treatment. Solid bars (■), *S. typhimurium*; Open bars (□), GBS. Relative percent internalization was calculated as follows: [(Number of CFU invaded into treated cells/Number of CFU invaded into control cells) X 100%]. Graphical representations are illustrative of the mean one representative experiment in which each assay condition was performed in triplicate. Error bars represent standard error of the mean. "*" indicates a *P* value < 0.05 compared to untreated control cells as evaluated using Dunnet's Multiple Comparisons Test.

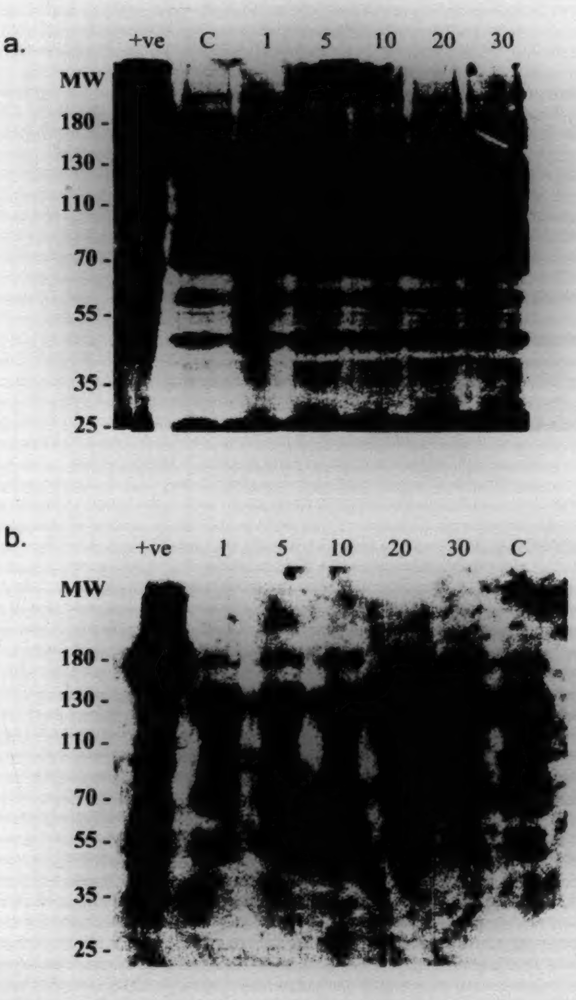
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Fig. I.4. (a). Immunoblot for host cell tyrosine phosphorylation. HeLa cells were either left untreated, (C; control cells), or infected with GBS for 1, 5, 10, 20, or 30 min. Cell lysates were resolved by 10% SDS-PAGE, blotted onto a nitrocellulose membrane, probed with anti-phosphotyrosine clone 4G10, and detected using ECL. The positive control (+ve) is an EGF-stimulated A431 cell lysate. (b). Immunoblot for tyrosine phosphorylation following immunoprecipitation of lysates collected as in (a). This does not reveal any further obvious alteration in the cellular tyrosine phosphorylation pattern in response to GBS infection.

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